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# Calcium homeostasis in Escherichia coli

Muhammad Arif

*University of New Hampshire, Durham*

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CALCIUM HOMEOSTASIS IN *ESCHERICHIA COLI*

BY

MUHAMMAD ARIF

B.S., Winona State University (Minnesota), 1994

M.S., Mississippi State University, 1997

DISSERTATION

Submitted to the University of New Hampshire

in Partial Fulfillment of

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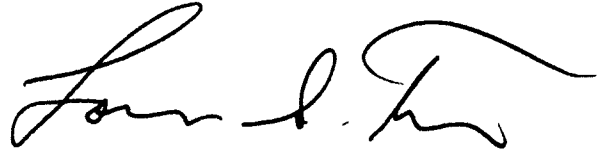
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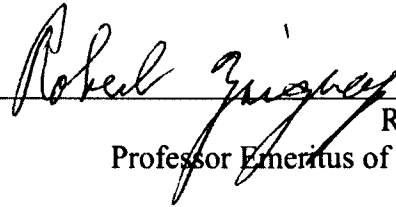


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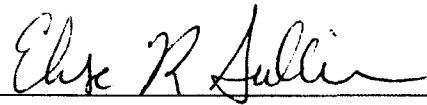
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Dissertation Director, Louis S. Tisa,  
Professor of Microbiology and Genetics



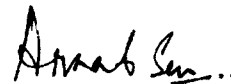
Robert Zsigray  
Professor Emeritus of Microbiology



Elise Sullivan  
Clinical Assistant Professor of Microbiology



Thomas G. Pistole  
Professor of Microbiology



Arnab Sen, Professor of Microbiology  
University of North Bengal, India

Head  
Department of Botany  
University of North Bengal

2/1/12  
Date

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## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	iii
LIST OF TABLES .....	vii
LIST OF FIGURES .....	viii
ABSTRACT .....	x
1. INTRODUCTION .....	1
Calcium and Prokaryotic Cell Function .....	2
Calcium Transport in Prokaryotes .....	4
Calcium and Bacterial Behavior .....	6
Mutants that are Defective in Calcium Transport.....	7
Mutants that are resistant to calcium channel blockers .....	10
The Use DNA Arrays for Functional Genomic Studies .....	11
Goal of the Research Project .....	11
What genes are globally regulated by calcium? .....	12
What happens to gene expression in mutants that are defective in calcium transport?.....	12
2. MATERIALS AND METHODS.....	14
Strains, plasmids, and phages .....	14
Growth Conditions.....	14

Transposon <i>TnphoA</i> and Mutagenesis of CC118 .....	16
Selection of Calcium-Sensitive Mutants .....	16
Calcium-Sensitivity Assay.....	17
Cation Sensitivity Assay .....	18
Chemotaxis Assay .....	18
Analysis of free-swimming behavior.....	19
Alkaline Phosphatase Assay .....	19
DAPI Experiments.....	20
Arbitrarily Primed PCR .....	21
Bacterial Growth Conditions for Panorama DNA Macroarray Experiments .....	23
Synthesis of <sup>33</sup> P-labeled cDNA Probe .....	23
Hybridization with Panorama <i>E. coli</i> Gene Array .....	24
Data Acquisition by Phosphorimaging and Analysis of the Arrays .....	25
Bacterial Growth Conditions for qRT-PCR Experiments .....	25
RNA extraction.....	26
Reverse Transcriptase Reaction and cDNA Synthesis .....	26
qPCR/Real-Time PCR .....	27
 3. RESULTS AND DISCUSSION .....	 30
Isolation of Calcium-Sensitive Mutants of <i>E. coli</i> by <i>TnphoA</i> Mutagenesis .....	30
Physiological and Biochemical Properties of Calcium-Sensitive Mutants .....	31
Molecular Characterization of Calcium-Sensitive Mutants .....	34
Panorama <i>E. coli</i> Gene Array .....	46
qRT-PCR and Comparative Analysis of Expression of Genes of Interest .....	51

15 Genes of Interest .....	52
Relative Expression of Parental and Mutant Strains under Control Condition ..	54
Relative Expression of 3 Parental Wild Type Strains under Control Condition .....	54
Analysis of Individual Gene Expression .....	57
<i>atpD</i> Expression.....	59
<i>marB</i> Expression.....	60
<i>secA</i> and <i>yjeE</i> Expression .....	65
<i>fadB</i> Expression .....	65
<i>pstS</i> , <i>arcA</i> , and <i>rpoS</i> Expression.....	66
<i>ftsZ</i> Expression.....	67
<i>pqiB</i> Expression .....	68
<i>corA</i> Expression.....	68
<i>atoA</i> Expression .....	69
<i>yfaD</i> and <i>ybbO</i> Expression .....	70
<i>cheB</i> Expression.....	71
Future Directions .....	71
LIST OF REFERENCES .....	73



## LIST OF TABLES

TABLE	PAGE
Table 1. List of Bacterial Strains. ....	15
Table 2. Primers used in Arbitrarily-primed PCR and sequencing reaction.....	22
Table 3. Primers for qRT-PCR used in the study. ....	29
Table 4. Properties of calcium-sensitive mutants .....	38
Table 5. cal gene mutations identified by ARB-PCR.....	45
Table 6. Panorama macroarray gene expression profiles and fold change as compared to control condition .....	50

## LIST OF FIGURES

FIGURE	PAGE
Figure 1. The transposon <i>TnphoA</i> (Manoil and Beckwith, 1985) .....	32
Figure 2. Photomicrograph of wild type <i>E. coli</i> and calcium-sensitive mutants .....	36
Figure 3. The Cal134 mutant is defective in cell division, but not DNA replication .....	37
Figure 4. Alkaline Phosphatase activity by Pho43 is time dependent .....	39
Figure 5. Ca-sensitive mutants were sensitive to other divalent cations .....	40
Figure 6. MIC values for Zn in the Ca-sensitive mutants .....	41
Figure 7. MIC values for Mg <sup>2+</sup> in the Ca-sensitive mutants .....	42
Figure 8. PCR amplification of transposon <i>TnphoA</i> .....	43
Figure 9. PCR amplification of transposon <i>TnphoA</i> in Cal134 .....	44
Figure 10. <i>E. Coli</i> DNA Macroarray Protocol .....	48
Figure 11. Panorama <i>E. coli</i> gene array exhibiting global gene expression profiles of <i>E. coli</i> MG1655 following growth on E medium (control), E medium supplemented with 75mM calcium, and E medium supplemented with 10mM EGTA. ....	49
Figure 12. Relative expression of 15 genes under elevated Ca <sup>2+</sup> condition (75mM Ca) and depleted Ca <sup>2+</sup> condition (10mM EGTA) in MG1655 .....	53
Figure 13. Relative expression of parental (KBT) versus mutant strains (calC and calD) .....	55
Figure 14. Relative expression of parental (CC118) versus mutant strains (PhoC23 and Cal134) .....	56
Figure 15. Relative expression of parental wild type strains (MG1655, KBT, CC118) .....	58

Figure 16. Relative expression of parental (KBT) versus mutant strains (calC and calD) under elevated calcium conditions (75 mM Ca <sup>2+</sup> ) .....	61
Figure 17. Relative expression of parental (KBT) versus mutant strains (calC and calD) under depleted calcium conditions (10 mM EGTA).....	62
Figure 18. Relative expression of parental (CC118) versus mutant strains (PhoC23 and Cal134) under elevated calcium conditions (75 mM Ca <sup>2+</sup> ) .....	63
Figure 19. Relative expression of parental (CC118) versus mutant strains (PhoC23 and Cal134) under depleted calcium conditions (10 mM EGTA).....	64

## ABSTRACT

### CALCIUM HOMEOSTASIS IN *ESCHERICHIA COLI*

by

Muhammad Arif

University of New Hampshire, May, 2012

While the role of calcium ions as secondary chemical messengers has been well described in eukaryotic cells, little is known about calcium homeostasis in bacteria at the physiological and molecular level. No calcium influx gene has been identified so far. This dissertation focuses on calcium regulation of gene expression by (i) determining the effects of elevated and depleted calcium levels on global gene expression in wild-type cells (MG1655), (ii) employing transposon mutants (calcium-sensitive) and mutants that are defective in calcium transport, and (iii) performing quantitative analysis of a specific subset of 15 genes to elucidate their possible involvement in calcium transport and homeostasis. The results of the systematic research approach corroborate existing knowledge regarding the involvement of genes *ftsZ*, *atpD*, *cheB*, *atoA* in calcium regulation. Quantitative real time PCR data revealed very high upregulation of genes *yjeE* and *secA* indicating their possible role as overactive calcium efflux systems in *E. coli*. My data also raises the distinct possibility of calcium's involvement in stress response as evidenced by upregulation of *marB* in transposon mutants and by 10-fold induction of *rpoS* in elevated calcium condition in mutants defective in calcium transport. The results

also reveal calcium-regulation of *yfaD* and *ybbO*, however, their functions are yet unknown. Cumulatively, a comprehensive picture emerges wherein it seems likely that calcium homeostasis represents a dynamic state with hitherto unknown regulatory activity throughout *E. coli* genome.

## CHAPTER 1

### INTRODUCTION

Calcium ions serve as secondary chemical messengers in eukaryotic cells and tissues including muscle, neural, and cardiovascular tissue. Calcium is also essential for cell cycle regulation, cellular differentiation, and movement (Smith, 1995; Norris *et al.*, 1996). Within prokaryotes, calcium participates in spore formation (Hogarth and Ellar, 1978), chemotaxis (Ordal, G.W., 1977; Tisa and Adler, 1992), motility, cell division, and signal transduction (Norris *et al.*, 1996).

*Escherichia coli* tightly regulates cytosolic free calcium at approximately 100nM, a level similar to that of eukaryotic cells (Gangola and Rosen, 1987). The mechanism of calcium entry is unknown. Four genes (*calA*, *calC*, *calD*, and *chaA*) have been identified in *E. coli* that are involved in calcium homeostasis. Recently, ATP has been proposed to regulate calcium efflux in *E. coli* through an ATPase (Naseem *et al.*, 2009). A knockout of the *atpD* gene (codes for a component of the F<sub>0</sub>F<sub>1</sub> ATPase) caused a defect in calcium efflux as measured by cytosolic free calcium levels. The defect in the  $\Delta$ atpD knockout cells was also found to exhibit a 70% reduction in ATP. Addition of 1 mM glucose or 1 mM methylglyoxal raised ATP in the  $\Delta$ atpD knockout cells to that of the wild type and restored calcium efflux.

In eukaryotic cells, calcium ions play an important role as a secondary messenger for signal transduction, behavior, and differentiation (Rasmussen and Rasmussen, 1990).

Evidence indicating that calcium ions also play a role in bacterial behavior and signal transduction is well established (Tisa and Adler, 1992, Tisa and Adler, 1995a, Tisa and Adler, 1995b, Tisa *et al.*, 1993, Tisa *et al.*, 2000).

### **Calcium and Prokaryotic Cell Function**

The role of calcium ions in prokaryotic cells is not as well defined as its role in eukaryotic cells. However, there is a growing body of evidence that shows an involvement of calcium in a variety of prokaryotic cell functions including microbial development, nitrogen fixation, signal transduction and the cell cycle (Norris *et al.*, 1991, Norris *et al.*, 1996, Onek and Smith, 1992, Smith, 1995).

Electron probe microanalysis and x-ray mapping of *E. coli* have revealed the total cellular  $\text{Ca}^{2+}$  content and its distribution (Chang *et al.*, 1986).  $\text{Ca}^{2+}$  was located predominately in the cell envelope. During cell division, an increase in cytoplasmic calcium was observed. It is not clear from this study whether this change in cytoplasmic calcium content represents an increase in free-  $\text{Ca}^{2+}$ . The  $\text{Ca}^{2+}$  levels of the cell envelope also increased during cell division. Norris and co-workers (Norris *et al.*, 1988) have proposed that prokaryotic cell cycle events are regulated through a single  $\text{Ca}^{2+}$  flux. It has also been suggested that membrane-bound  $\text{Ca}^{2+}$  mediates physiological or pathobiological changes in *E. coli* (Boulanger *et al.*, 1985, Elsbach *et al.*, 1985, Guzman *et al.*, 1991). Furthermore,  $\text{Ca}^{2+}$ -stimulated kinase activities have been found in *E. coli* (Nakashima *et al.*, 1992, Rampersaud *et al.*, 1991, Sweeney *et al.*, 1995) and other

bacteria (Sandler and Keynen, 1992, Stowe *et al.*, 1989, Tisa *et al.*, 2000). These studies imply that calcium plays a variety of regulatory roles in bacteria.

Ca<sup>2+</sup>-binding proteins and calmodulin-like proteins have been reported to be present in a wide variety of bacteria (Onek and Smith, 1992). For example, a calcium-binding protein with a structure similar to EF-hand calcium-binding proteins that are found in eukaryotic cells (Heizmann and Hunziker, 1991) has been identified in *Streptomyces erythreus* (now called *Saccharopolyspora erythraea*) (Leadley *et al.*, 1984, Swan *et al.*, 1989). It was purified to homogeneity (Leadley *et al.*, 1984,) and the structural gene was cloned and sequenced (Swan *et al.*, 1989). While the protein clearly binds Ca<sup>2+</sup> (Bylsma *et al.*, 1992), its function is unknown. However, the striking structural similarity to proteins involved in Ca<sup>2+</sup>-regulation in eukaryotes raises tantalizing evolutionary questions. Calmodulin-like proteins have not been as well characterized in other bacterial systems. Immunological evidence indicates the presence of three calmodulin-like proteins in *E. coli*, which were suggested to be involved in calcium ion regulation (Laoudi *et al.*, 1994). The actual role of these proteins in calcium homeostasis and other cellular functions is unknown and warrants further study.

Extracellular calcium controls cPHB [complexed poly-(R)-3-hydroxybutyrate] biosynthesis through the AtoS-AtoC two-component system (Theodorou and Kyriakidis, 2009). Increased cPHB accumulation occurs at higher extracellular Ca<sup>2+</sup> concentrations in AtoS-AtoC-expressing *E. coli* compared to  $\Delta$ *atoSC* mutants. Downregulation of cPHB biosynthesis in the presence of EGTA is circumvented by the addition of calcium and magnesium. The calcium channel blocker verapamil reduces total and membrane-bound



cPHB levels, but this inhibitory effect is reversed by the addition of calcium to cells with a functional AtoS-AtoC two-component.

### **Calcium Transport in Prokaryotes**

*E. coli* regulates and maintains a low level of cytosolic free- $\text{Ca}^{2+}$  (90 nM) similar to the level observed with eukaryotic cells (Gangola and Rosen, 1987, Knight *et al.*, 1991, Tisa and Adler, 1995a ). These levels cytosolic free- $\text{Ca}^{2+}$  in cells of *E. coli* have been directly measured by the use of fura-2, a fluorescent  $\text{Ca}^{2+}$ -indicator dye (Gangola and Rosen, 1987, Tisa and Adler, 1995a) and by the use of aequorin,  $\text{Ca}^{2+}$ -sensitive photoprotein (Knight *et al.*, 1991). It is presumed that all bacteria regulate and maintain their cytosolic  $\text{Ca}^{2+}$  at similar low levels.

Calcium transport mechanisms have been found in all bacterial systems examined to date (Rosen, 1986). There are two calcium efflux systems in *E. coli*: a  $\text{Ca}^{2+}/\text{PO}_4^{2-}$  symporter and a  $\text{Ca}^{2+}/\text{H}^+$  antiporter. The mechanism of  $\text{Ca}^{2+}$  entry is unknown. Several calcium-sensitive mutants have been isolated, and three different calcium-sensitive loci were identified: *calA*, *calC*, and *calD* (Brey and Rosen, 1979). *CalB* was also identified, but this mutant strain was not maintained. All three of these loci, A, C, and D, are associated with  $\text{Ca}^{2+}/\text{PO}_4^{2-}$  symporter activity. Mutations in either the *calC* or *calD* loci result in defective  $\text{Ca}^{2+}/\text{PO}_4^{2-}$  symporter activity. The *calA* mutation results in an increase in  $\text{Ca}^{2+}/\text{PO}_4^{2-}$  symporter activity. The *calA* phenotype is expressed in cells only in a *corA* ( $\text{Co}^{2+}$  resistance) background, however the *calA* locus by itself will cause an increase in  $\text{Ca}^{2+}/\text{PO}_4^{2-}$  symporter activity. *CorA* mutants are defective in one of the  $\text{Mg}^{2+}$  transport

systems and are sensitive to  $\text{Ca}^{2+}$  (Park *et al.*, 1976). The presence of *calA* in a *corA* background increased their sensitivity to  $\text{Ca}^{2+}$ . *CorA* mutants are sensitive to 50 mM  $\text{Ca}^{2+}$ , while a *calA corA* double mutant is sensitive to 25 mM  $\text{Ca}^{2+}$  (Brey and Rosen, 1979). A fourth  $\text{Ca}^{2+}$  transport gene, *chaA*, was identified, cloned, and sequenced (Ivey *et al.*, 1993). An operon containing *chaA* and two other *cha* genes have been identified (Blattner *et al.*, 1997). The *chaA* gene is involved in  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter activity.

Naseem and colleagues (2009) showed the necessity for ATP during  $\text{Ca}^{2+}$  efflux. A global genomic expression profile of *E. coli* with elevated cytosolic calcium yielded candidate  $\text{Ca}^{2+}$ -regulated genes (for transport and membrane proteins). Subsequently a select few mutants from Keio knockout collection were tested and the researchers found a knockout of *atpD*, coding for a component of the  $\text{F}_0\text{F}_1$  ATPase, as defective in  $\text{Ca}^{2+}$  efflux. The defect in the *atpD* knockout cells was accompanied by a 70% reduction in ATP. One millimolar glucose or 1 mM methylglyoxal raised ATP in the knockout mutant to that of the wild-type and restored  $\text{Ca}^{2+}$  efflux. Furthermore, 1mM 2,4-dinitrophenol lowered the ATP in wild-type cells and resembled the depleted levels in the *atpD* knockout cells. Concomitantly to lower ATP level, a similar defect in  $\text{Ca}^{2+}$  efflux in wild-type was observed as in *atpD* knockout mutants. Addition of 10mM  $\text{Ca}^{2+}$  resulted in a 30% elevation in ATP in wild-type cells. They proposed that ATP is most likely to regulate  $\text{Ca}^{2+}$  efflux in *E. coli* through an ATPase.

### **Calcium and Bacterial Behavior**

Motile bacteria such as *E. coli* perform chemotaxis by moving toward chemical attractants and away from chemical repellents (Blair, D.F., 1995). Each bacterium possesses the following: (i) a sensory apparatus that recognize chemical signals, (ii) a signal transduction mechanism, (iii) a pathway to conduct messages to the flagella, and (iv) a response mechanism that modifies the direction of the flagella that differentiates the response to an attractant (running) from the response to a repellent (tumbling).

Bacterial cells sense chemical gradients using transmembrane receptors called methyl-accepting chemotaxis proteins [MCPs] (Blair, D.F., 1995). There are four known MCPs in *E. coli*. The cytoplasmic proteins CheW, CheA, CheY, and CheZ employ a phosphorylation cascade to transmit excitatory signals from the receptors to the flagellar motor. Attenuation of signals during a continued presence of the stimulus is initiated by cytoplasmic proteins CheR and CheB, which are involved in the adaptation process by methylation and demethylation, respectively, of the MCPs.

The role of calcium ion participation in bacterial behavior and signal transduction has been established by several lines of evidence (Tisa and Alder, 1992, Tisa and Alder, 1995a, Tisa and Alder, 1995b, Tisa *et al.*, 1993, Tisa *et al.*, 2000, Watkins *et al.*, 1995). Previous studies demonstrate the following observations: (1) Changing cytoplasmic free  $\text{Ca}^{2+}$  levels in cells of *E. coli* alters their behavior (Tisa and Alder, 1992). Tumbling ensues following the release of free  $\text{Ca}^{2+}$  that is produced from caged  $\text{Ca}^{2+}$  compounds such as nitr-5 upon irradiation. (2) Changes in bacterial behavior are correlated with changes in cytoplasmic free  $\text{Ca}^{2+}$  level (Tisa and Alder, 1995b). Fluorescent  $\text{Ca}^{2+}$

indicator dye fura-2 was used to show that repellents caused a temporary rise in cytoplasmic free  $\text{Ca}^{2+}$  levels, while attractants caused a temporary fall, and the receptor proteins were required for these effects. (3) Some mutants defective in calcium transport have elevated levels of cytoplasmic free  $\text{Ca}^{2+}$ , tumble continuously, and are defective in chemotaxis (Tisa and Adler, 1995a). (4) Calcium ion channel blockers inhibit *E. coli* chemotaxis (Tisa *et al.*, 1993, Tisa *et al.*, 2000).

### **Mutants that are Defective in Calcium Transport**

Brey and Rosen (Brey and Rosen, 1979) isolated three calcium-sensitive mutants, calA, calC, and calD that are defective in  $\text{Ca}^{2+}/\text{PO}_4^{2-}$  symporter activity. These mutants provide useful tools to investigate calcium homeostasis.

Fluorescent  $\text{Ca}^{2+}$ -indicator dyes such as fura-2, indo-1 or fluo-3, have been extensively used as tools in cell biology to study intracellular free  $\text{Ca}^{2+}$  levels in eukaryotic cells (Thomas and Delaville, 1991). Cytoplasmic free  $\text{Ca}^{2+}$  levels were measured in wild type and mutant strains by electroporating the fluorescent  $\text{Ca}^{2+}$ -indicator dyes fura-2 or fluo-3 into *E. coli* (Tisa and Adler, 1995a). The technique enabled Tisa and Adler (1995) to carry out the first direct measurement of cytosolic free  $\text{Ca}^{2+}$  levels in *E. coli* mutants defective in calcium transport (calA, calC, and calD). Chemotactically wild-type cells exhibited a cytosolic free-  $\text{Ca}^{2+}$  level of about 105 nM which is similar to the value of  $90 \pm 10$  nM reported by Gangola and Rosen (Gangola and Rosen, 1987). The cytosolic free  $\text{Ca}^{2+}$  levels of the mutants were approximately 90 nM, 1130 nM, and 410 nM for calA, calC, and calD, respectively (Tisa and Adler, 1995a).

The high levels of  $\text{Ca}^{2+}$  in calC and calD were responsible for these mutants to be tumbling (Tisa and Adler, 1995a). In the absence of any added stimuli, both the calC and the calD mutants swam with a tumbling bias and both of the cal mutants were defective in chemotaxis.

Wild-type bacteria tightly regulate intracellular free- $\text{Ca}^{2+}$  at about 100 nM, similar to the level observed in eukaryotic cells (Gangola and Rosen, 1987, Tisa and Adler, 1995a). Treating cells with EGTA consequently reduced total cellular content of  $\text{Ca}^{2+}$  but had no effect on intracellular free- $\text{Ca}^{2+}$  concentration. While elevating external  $\text{Ca}^{2+}$  increased total cellular  $\text{Ca}^{2+}$  content, it had no effect on the intracellular free- $\text{Ca}^{2+}$  level. However, the cytoplasmic  $\text{Ca}^{2+}$  levels of the calC and calD mutants were influenced by external calcium. These mutant cells when grown in the presence of EGTA, a  $\text{Ca}^{2+}$ -chelator, had lower cytoplasmic  $\text{Ca}^{2+}$  levels than those cells grown in the absence of EGTA. The addition of calcium to the growth medium resulted in elevated levels of cytoplasmic  $\text{Ca}^{2+}$  levels for these two *cal* mutants. These results indicate that cytoplasmic  $\text{Ca}^{2+}$  levels are not regulated in the calC and calD mutants.

Although several calcium channel blockers inhibit *E. coli* chemotaxis,  $\omega$ -conotoxin has been shown to be the most effective agent (Tisa *et al.*, 1993, Tisa *et al.*, 1999). Cells treated with  $\omega$ -conotoxin swim at a normal rate with a smooth basis, i.e., tumbling is blocked.  $\omega$ -Conotoxin does not affect the  $\text{Ca}^{2+}$  efflux systems  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter or  $\text{Ca}^{2+}/\text{PO}_4^{4-}$  symporter activity (Tisa *et al.*, 1993). The two chemotactically-defective calcium-sensitive mutants, calC and calD (Tisa and Adler, 1995a) were also tested.  $\omega$ -Conotoxin slightly inhibited residual chemotaxis by the calD mutant and

slightly stimulated the residual chemotaxis by the calC mutant. These results suggest that  $\omega$ -conotoxin may be impeding calcium entry in the calC mutant and reducing its cytoplasmic free  $\text{Ca}^{2+}$  level. The resultant reduction of  $\text{Ca}^{2+}$  would lead to the partial recovery of its chemotactic behavior.

The calA mutant is relatively resistant to the effect of  $\omega$ -conotoxin. This mutant requires a 10-fold higher  $\omega$ -conotoxin concentration for same level of inhibition as compared to the wild type (Tisa *et al.*, 1993). There are two models that explain  $\omega$ -conotoxin resistance in calA: [1] The mutation results in alteration of the  $\omega$ -conotoxin binding site. [2] Alternatively, calA affects some process or site downstream from the binding site. To distinguish between these two models, the kinetics of  $^{125}\text{I}$ - $\omega$ -conotoxin binding to right-side-out membranes from both wild type and calA-mutant cells was investigated (Tisa, 2000). The results indicate that  $^{125}\text{I}$ - $\omega$ -Conotoxin binds to right-side-out membrane vesicles with saturation kinetics. Binding of  $^{125}\text{I}$ - $\omega$ -conotoxin to membrane vesicles was inhibited by  $\text{Ca}^{2+}$  ions, but not by  $\text{Mg}^{2+}$  ions. These results imply that a specific binding site(s) exists on the cytoplasmic membrane. Analysis of the kinetics of  $^{125}\text{I}$ - $\omega$ -conotoxin binding to membrane vesicles indicates that both the wild type and the calA mutant had a similar  $K_D$  for  $\omega$ -conotoxin but the saturation level was higher with the calA mutant. These results indicate that there are more binding sites in the calA mutant, but these binding sites have the same affinity as the wild type. Thus, calA does not directly affect the affinity of the  $\omega$ -conotoxin binding site. Chemical cross-linking experiments showed two proteins as potential  $\omega$ -conotoxin receptors.

### **Mutants that are resistant to calcium channel blockers**

I.B Holland and associates (Bouquin *et al.*, 1995, Casarégola *et al.*, 1991; Chen *et al.*, 1991) isolated *E. coli* mutants that are specifically resistant to calcium channel inhibitors, such as verapamil and diltiazem. The parental cells were hypersensitive to many drugs. Holland proposed that cells, which are resistance to  $\text{Ca}^{2+}$  channel inhibitors, have altered or poorly regulated intracellular concentration of free  $\text{Ca}^{2+}$  (Blattner *et al.*, 1997). However, he did not measure their calcium content. Tisa (unpublished data) measured and found these mutants also have slightly elevated cytoplasmic free- $\text{Ca}^{2+}$  levels (about 150 to 200 nM) compared to the wild type (about 100 nM), but these levels were not as high as those observed with the calC and calD mutants (400 to 1,300 nM range).

With these verapamil and diltiazem resistant mutants Laoudj *et al* identified calcium-regulated proteins (Laoudi *et al.*, 1997). Treating cells with the  $\text{Ca}^{2+}$  chelators, EGTA and BAPTA, resulted in induction of at least 25 polypeptides. The synthesis of at least 11 polypeptides was repressed under these conditions. These effects were also detected in the parental wild type but they were more dramatic in the mutant strains. Since this pattern of induction was not observed in heat- or SDS-treated cells, it does appear to be a general stress response. Three of the induced major polypeptides cross-reacted with polyclonal and monoclonal antibodies to calmodulin or calerythrin.

## **The Use DNA Arrays for Functional Genomic Studies**

The use of DNA arrays for genome-wide gene expression has been employed in many applications including drug discovery (Debouck and Goodfellow, 1999; Marton *et al*, 1998), measurement of gene copy number (Pinkel *et al*, 1998), discovery of disease-related genes in humans (DeRisi *et al*, 1996), gene mapping (Chee *et al*, 1996) and gene expression (DeRisi *et al*, 1997; Holstege *et al*, 1998; Lashkari *et al*, 1997; Schena *et al*, 1995; Spellman *et al*, 1998).

In a landmark paper, Tao and colleagues (Tao *et al*, 1999) used the Panorama *E. coli* Gene Array (Sigma-Genosys Biotechnologies, Inc.) to publish a functional genomics study on bacteria. They successfully used cDNA arrays that contained all 4,290 open reading frame (ORF)-specific DNA fragments from *E. coli* K-12 (MG 1655) to analyze differential expression profiles of *E. coli* grown on rich and on minimal media. The genome-wide data not only corroborated the results obtained from numerous individual physiology experiments conducted over the years, but several genes were assigned to functional groups on the basis of co-regulation with similar or related genes. From their data they also developed several testable hypotheses about bacterial metabolism and their experiments demonstrated the potential of functional genomics.

## **Goal of the Research Project**

Our current calcium regulated model system includes calcium-sensitive *TnphoA* mutants of *E. coli* that we have isolated and have physiologically characterized. It also includes *calC* and *calD* mutants that have elevated cytoplasmic calcium since they cannot



regulate cytoplasmic calcium levels due to a defect in calcium transport. A system such as the one we have, which encompasses cytoplasmic calcium levels from very low to very high, makes it an ideal candidate to study calcium regulation of gene expression.

The specific aims of the project are the following:

### **What genes are globally regulated by calcium?**

Panoramic™ *E. coli* DNA macroarrays will be used to identify those genes that are globally regulated by calcium. I am interested in determining the effects of elevated and depleted calcium levels on global gene expression in wild-type cells, which maintain a steady-state level of cytoplasmic calcium. The results of these experiments will help answer the question: What genes are involved in calcium homeostasis?

### **What happens to gene expression in mutants that are defective in calcium transport?**

The expression profile of mutants that are defective in calcium-transport will also be determined under the same elevated and depleted calcium conditions. The calC and calD mutants do not regulate cytoplasmic calcium levels. In the presence of elevated calcium, their cytoplasmic calcium levels increase, while in the presence of EGTA (calcium chelator) their cytoplasmic calcium levels decrease (Tisa and Adler, 1995a). Since the cytoplasmic calcium level in these mutants is not regulated, these experiments will determine if and how cytoplasmic calcium affects gene expression.

In addition to the above-mentioned goals, it is noteworthy to repeat that no Ca<sup>2+</sup> influx gene has been identified so far. My goals also include the search for such influx

gene candidates while simultaneously broadening the investigation to include assessment of a few genes that are listed in *E. coli* genome databases possessing “unknown function.” The four known calcium homeostasis genes are not clustered but map at 95.0, 15.2, 9.4, 27.4 min for *calA*, *calC*, *calD*, and *chaA*, respectively. It is indeed very likely that calcium homeostasis includes a lot more genes. Identification of new genes that are regulated by calcium is one of my major goals in my approach to understand calcium homeostasis in *E. coli*.

The systematic research approach will initially center on generating calcium-sensitive transposon mutants and characterization of these mutants, followed by an array analysis of the sequenced *E. coli* MG1655 under elevated and depleted calcium conditions to generate a list of probable *cal* genes that are affected by  $\text{Ca}^{2+}$ , then focusing on several of these genes and performing a more stringent quantitative gene expression analysis to reveal their potential relationship regarding calcium homeostasis.

## CHAPTER 2

### MATERIALS AND METHODS

#### **Strains, plasmids, and phages**

All strains of *E. coli* used in this study are derivatives of K12 and are listed in Table 1.

#### **Growth Conditions**

*E. coli* K12 strain CC118 ( $\Delta$ *phoA*, spectinomycin resistant) was the parental strain that was used for mutagenesis and subsequent generation of calcium-sensitive mutants. Cells were grown and maintained in LB medium containing the appropriate antibiotics. The calcium-sensitive mutants have been designated: Cal117, Cal512, Pho43, Cal102, Cal134, PhoA4, PhoC23, Cal504, Cal719, and Cal526. For cation sensitivity assays, cells were grown in E medium at a pH of 7.8 (Brey and Rosen 1978). E medium (0.5% bactopectone with 120mM Tris, 70mM NaCl, 20mM KCl, 20mM NH<sub>4</sub>Cl, 3mM Na<sub>2</sub>SO<sub>4</sub>, 1mM MgCl<sub>2</sub>, 2 $\mu$ M ZnCl<sub>2</sub>, and 0.4% glycerol) offers an advantage due to the fact that it is a low phosphate medium, which allows the addition of supplemental cations without precipitation of solid phosphate salts. For motility and chemotaxis assays, cells were grown in tryptone broth consisting of 1% Bacto-tryptone (Difco Laboratories) and 0.5% NaCl and incubated at 35<sup>0</sup>C. In some cases, cells were grown in Vogel-Bonner medium

Table 1. List of Bacterial Strains.

Strain	Phenotype	Reference or Source
CC118	araD139 $\Delta$ (ara,leu)7697 $\Delta$ lacX74 phoA $\Delta$ 20 galE galK thi rpsE rpoB argEam recA1	Colin, M and J. Beckwith 1985
KBT001	Chemotactically wild type purE trp leu proC ara lac rpsL metE lysA	Brey and Rosen, 1979
Cal102	CC118::TnphoA	This Study
Cal117	CC118::TnphoA	This Study
Cal134	CC118::TnphoA	This Study
Cal512	CC118::TnphoA	This Study
Cal504	CC118::TnphoA	This Study
Cal719	CC118::TnphoA	This Study
Cal526	CC118::TnphoA	This Study
Pho43	CC118::TnphoA	This Study
PhoA4	CC118::TnphoA	This Study
PhoC23	CC118::TnphoA	This Study
RB063	calC	Brey and Rosen, 1979
RB073	calD	Brey and Rosen, 1979
MG1655	Wildtype K12 strain, sequenced first.	Blattner <i>et al.</i> , 1997
$\lambda$ TnphoA	Tn5 IS50L::phoA (KmR)	Gutierrez et al, 1987

(Vogel and Bonner, 1956) containing the required amino acids at 1 mM and 50 mM glycerol (minimal glycerol medium) or 50 mM DL-lactate (minimal lactate medium).

### **Transposon *TnphoA* and Mutagenesis of CC118**

The transposon *TnphoA* was introduced into strain CC118, the parental wild type *E. coli* strain, by phage  $\lambda$  infection ( $\lambda$  *TnphoA*) according to the method of Manoil and Beckwith (1985) at a multiplicity of infection (MOI) of 0.05. Cells of strain CC118 were incubated at 37°C in LB medium containing 10 mM MgSO<sub>4</sub> and 0.4% maltose. Overnight grown cells were harvested by centrifugation and resuspended in 10 mM MgSO<sub>4</sub>. The washed cells were infected with  $\lambda$  *TnphoA* lysate as described in the next section.

### **Selection of Calcium-Sensitive Mutants**

For one series, each 100  $\mu$ l sample of cells was infected with 10, 100, 200, or 500  $\mu$ l of lysate. The mixture was incubated at 30°C for 20 min. Following the addition of 1 ml of LB medium, the cells were allowed to outgrow for 30 min at 30°C. The cells were harvested by centrifugation at 6,000 x g for 10 min and resuspended in E medium containing glycerol (glycerol-E medium). This procedure was repeated twice and the cells were finally resuspended in 1 ml of glycerol-E medium containing 50 mM CaCl<sub>2</sub> and 60  $\mu$ g/ml kanamycin. Following an incubation at 35 °C for 1 hr, penicillin G (15,000 U) was added and the cells were allowed to incubate for 3 hr. Penicillin G was removed by centrifugation at 6,000 x g for 10 min and resuspending the cell pellet in glycerol-E

medium. This washing step was repeated twice. The cells were finally resuspended in 1 ml of glycerol-E medium and 100- $\mu$ l samples were plated on glycerol-E medium plates containing 5 mM  $\text{CaCl}_2$ , 60  $\mu\text{g/ml}$  kanamycin and 40  $\mu\text{g/ml}$  5-bromo-4-chloro-3-indolyl phosphate (XP) to screen for  $\text{TnphoA}$  insertions exhibiting PhoA activity. The plates were incubated at 37°C for 2 days before colonies were picked.

For the second series, a 100-  $\mu$ l sample of cells was infected with 100-  $\mu$ l of phage lysate for 20 min at 37°C and directly plated on either (1) glycerol-E medium containing 50 mM  $\text{CaCl}_2$ , 30  $\mu\text{g/ml}$  kanamycin and 40  $\mu\text{g/ml}$  XP or (2) glycerol-E medium containing 5 mM  $\text{CaCl}_2$ , 180  $\mu\text{g/ml}$  kanamycin and 40  $\mu\text{g/ml}$  XP. These plates were also incubated at 37°C for 2 days before colonies were picked.

Atypically small colonies on the plates were judged to be potential calcium-sensitive mutants. Those colonies that grew on glycerol-E medium but not on glycerol-E medium containing 50 mM  $\text{CaCl}_2$  (thus indicating sensitivity to calcium) were purified and retained.

### **Calcium-Sensitivity Assay**

Calcium-sensitivity was later defined as the inability to grow in the presence of 75 mM  $\text{CaCl}_2$ . Calcium-sensitivity was measured by two different growth inhibition assays.

Initially, calcium-sensitivity was measured by growth inhibition assay of Brey and Rosen (1979). Cells were screened for their ability to grow on E medium plates supplemented with 0mM, 25 mM, 50mM, 75 mM, or 100mM  $\text{CaCl}_2$ . The plates were incubated at 37°C and the growth results were monitored at 24 hr and 48 hr. After 48 hr,

calcium-sensitive cells are unable to grow on E medium plates supplemented with 75 mM CaCl<sub>2</sub>.

Growth inhibition was also determined from turbidity measurements in broth cultures. During this assay, cells were inoculated in E medium broth supplemented with glycerol and different concentrations (0mM, 25 mM, 50mM, 75 mM, or 100mM) of CaCl<sub>2</sub>, and were incubated at 37°C for 8 hr. The optical density at a wavelength of 600 nm was measured at 0 and 8 hrs.

#### **Cation Sensitivity Assay**

The sensitivity to various other cations was monitored by growth inhibition assay of the calcium-sensitive cells as described by Brey and Rosen (1979). The minimum inhibitory concentration (MIC) values were determined for each cation tested. MIC is the lowest concentration of cation at which all bacterial growth is stopped.

#### **Chemotaxis Assay**

Chemotactic ability was measured by swim-ring migration assay (Adler 1966). In this assay, bacteria migrate in response to a gradient of amino acids created by their metabolism. Wild type *E. coli* cells form two or more chemotactic rings on a tryptone swarm plate. The outer ring represents L-serine chemotaxis and the inner ring represents L-aspartate chemotaxis. Chemotactic properties are assessed on the spreading of the rings on the plates. Tryptone swarm plates containing 1 % Bacto-tryptone, 0.5 % NaCl, and 0.25 % Bactoagar (Difco Laboratories) were inoculated with a stab of approximately 10<sup>6</sup>

cells at the center of the plates and incubated at 30°C for 8 to 14 hr. The ring diameters were measured at the end of the experiment.

### **Analysis of free-swimming behavior**

Bacterial swimming behavior was observed at 30°C by phase-contrast microscopy at a magnification of 400x. The cells in these behavioral assays were suspended in chemotaxis medium (10 mM potassium phosphate pH 7.0, 0.1 mM potassium EDTA, and 1 mM L-methionine) or in filtered spent/used growth medium to an optical density at 590 nm of 0.1. The microscopic behavior was videotaped and analyzed by computer (Sager et al 1988).

### **Alkaline Phosphatase Assay**

Alkaline phosphatase activity was measured using both solid (for all mutants) and liquid media (only for calcium-sensitive transposon mutant Pho43). Cells were grown at 37°C in LB medium containing appropriate antibiotics. Overnight cultures were then streaked for isolation on E medium plates containing the chromogenic alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). XP is a substrate for alkaline phosphatase. The parental strain CC118 lacks functional alkaline phosphatase. Only colonies that produced alkaline phosphatase fusion on the outside of the cell or in the periplasmic space would turn blue. Following overnight growth at 37°C, successful calcium-sensitive mutants with functional alkaline phosphatase were monitored for blue color production.



For quantitative assays, Pho43 cells were incubated overnight in 10 mL tryptone broth (without kanamycin for wildtype, with kanamycin for mutants) 37°C. The following day, 1 mL of overnight culture was added to 10 mL of fresh tryptone broth (with or without calcium and kanamycin). After incubation at 37°C for 2 hr, cells were harvested and resuspended in 2.5 mL of 1M Tris-HCl (pH 8). The optical density of the cells was measured at 600 nm. A fraction (1 mL) of the cells was permeabilized by treatment with 0.1% SDS and chloroform (50  $\mu$ L each), while a 1 mL fraction was left untreated as a control. The 1 mL samples were incubated with 400  $\mu$ L of 0.4% p-nitrophenyl phosphate (PNPP) at 37°C for 20 min. The substrate in this reaction (PNPP) is hydrolyzed by alkaline phosphatase into a yellow colored product (maximal absorbance at 405nm). The reaction was stopped by the addition of 200  $\mu$ L of 1M NaHPO<sub>4</sub> at the end of 20 minutes. Reaction times were recorded with the use of stopwatches. OD<sub>405</sub> readings were taken. Alkaline phosphatase activity was assayed at different time intervals to optimize the reaction (Figure 7). One unit of alkaline phosphatase was defined as the amount of enzyme required to catalyze the hydrolysis of 1 nmol of PNPP per min at 37°C.

### **DAPI Experiments**

Prior to staining the cells were first fixed with toluene and treated with chloramphenicol to condense their genomes. The fixed cells were incubated with the fluorescent DNA binding dye DAPI (4', 6'-diamidino-2-phenylindole 2 HCl), and

observed by the use of phase contrast microscopy and fluorescence microscopy at a total magnification of 1000X.

### **Arbitrarily Primed PCR**

To identify the *cal* gene sequence, the region of the genes fused to the reporter gene (*phoA*) were amplified by PCR and sequenced. To amplify partial regions of *cal* genes fused to the transposon, we used the arbitrarily-primed PCR method (Caetano-Annoles, 1993; O'Toole and Kolter, 1998). This method involved two rounds of PCR amplification using arbitrary primers to prime from the chromosome and primers specific to *TnphoA*. During the first round of PCR, the primer (Tn-R) with sequence homology to the right end of the transposon *TnphoA* and arbitrary primer ARB1 were used. The PCR parameters for first round were as follows: (1) 95°C 5 min, (2) 6 cycles of 95°C for 30 s, 30°C for 30 s, 72°C for 1.5 min, (3) 30 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 2 min. During the second round of PCR, a 5 µL aliquot of first round PCR product was used as template and the primers ARB2 and Tn-I were used for amplification. The PCR parameters for second round were as follows: 30 cycles of 95°C for 30 s, 45°C for 30 s, 72°C for 2 min. The final PCR products were purified from an agarose gel with the aid of  $\beta$ -agarase. The primers (ARB1, ARB2, Tn-I and Tn-R) used in this experiment are listed in Table 2.

These PCR amplified products were sequenced using primers (*TnphoA*-int and Tn-R) located close to the fusion site on *TnphoA*. DNA sequence data were analyzed by sequence analysis software DNASTar for Mac. The resultant edited sequences, ORFs

Table 2. Primers used in Arbitrarily-primed PCR and sequencing reaction

Primer	Primer sequence (5' - 3')	Reference
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	This Study
ARB2	GGCCACGCGTCGACTAGTAC	This Study
Tn-I	CCTTCGGCATAATTACGTGC	This Study
Tn-R	GCAGTCTGATCACCCGTTAAA	This Study
TnphoA-int	TTTCCAGAACAGGGCAAAAC	This Study
TnphoA-F	TGCAAGTTGAAGGTGCGTCAATCG	This Study
TnphoA-R	ATGAGATGCCCTGCAAGCAATTCG	This Study

present in the edited sequences, as well as translated sequences were used to query the *E. coli* genomic database (BLAST at NCBI).

### **Bacterial Growth Conditions for Panorama DNA Macroarray Experiments**

To provide a comprehensive insight into calcium homeostasis, the expression profiles of wild type *E. coli* MG 1655 were determined under three different conditions: [1] growth medium containing elevated levels of calcium (the addition 75 mM CaCl<sub>2</sub>), [2] growth medium containing depleted levels of calcium (the addition of 10 mM EGTA) and [3] growth medium control (untreated). Overnight cultures of *E. coli* MG 1655 were used to inoculate fresh E media and the cultures were incubated at 37°C with rotary oration. For the array experiments cultures were grown to an OD<sub>600</sub> of 0.4 to 0.5 and then either 75 mM calcium or 10 mM EDTA was added. After 30 min of rotary aeration at 37°C, total RNA was quickly extracted by using Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA). Proper precautions were taken to avoid contamination with RNases and a non-denaturing electrophoresis gel was run with the extracted RNA to check the integrity of RNA. The flowchart depicted in Figure 10 details the overall process strictly adhered to during conducting the Panorama DNA macroarray experiments.

### **Synthesis of <sup>33</sup>P-labeled cDNA Probe**

Sigma-Genosys Biotechnologies, Inc. had developed commercially available cDNA primers that are specifically designed to preferentially label cDNA from mRNA. These C-terminal primer sets (4,290 ORF-specific C-terminal primers) were used to

generate the hybridization probe according to the manufacturers recommendations. As recommended by the manufacturer,  $^{33}\text{P}$ - $\alpha$ -dCTP was used to label the cDNA because, when compared to  $^{32}\text{P}$ , the signal from  $^{33}\text{P}$ -label cDNA yields better-defined spots and enhanced quantification of the signals. The  $^{33}\text{P}$  -labeled cDNA was purified and unincorporated-labeled nucleotides were removed by the use of Sephadex® G-25 gel-filtration spin columns. An estimation of percentage incorporation of  $^{33}\text{P}$  -dCTP into the cDNA was determined by using a hand-held Geiger counter or by scintillation counts of samples before and after column purification. The labeled cDNA probe was then ready to be used in a hybridization reaction with the Panorama *E. coli* Gene arrays (Sigma-Genosys Biotechnologies, Inc.).

#### **Hybridization with Panorama *E. coli* Gene Array**

Following its preparation and purification, the  $^{33}\text{P}$ -labeled cDNA was hybridized to the Panorama gene array by the use of roller bottles in a hybridization oven. The hybridization and washing steps were performed according to the protocol specified in Panorama *E. coli* cDNA Labeling and Hybridization Kit. The DNA array blots were rinsed in 2X SSPE (1X SSPE: 0.18 M NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , 1 mM EDTA, pH 7.7) and pre-hybridized in pre-warmed hybridization solution (5X SSPE, 2% SDS, 1X Denhardt's reagent, 100  $\mu\text{g}/\text{ml}$  sheared salmon sperm DNA) at 65°C for 1 hr at 6rpm. The entire  $^{33}\text{P}$ -labeled cDNA was first denatured at 95°C for 10 min and then added to 3 ml of hybridization buffer and the blot were hybridized in this solution for 15 hr at 65°C. After overnight incubation, the blots were washed with buffer (0.5X SSPE, 0.2% SDS) three

times at room temperature. The blots were then washed three more times with pre-warmed (65°C) buffer in the hybridization oven at 65°C for 20 min at 6 rpm. Washed blots were finally air-dried briefly for 5 min and wrapped in clear plastic food wrap and exposed to a phosphorimager screen (Bio-Rad Laboratories) for 24 h.

### **Data Acquisition by Phosphorimaging and Analysis of the Arrays**

The gene expression signals were measured by the use of a Bio-Rad PhosphorImager with a Kodak Low Energy Storage Phosphor Screen. Typically, the arrays were exposed overnight to yield quantifiable results. For quantification, imaging screens were scanned at a 50µm pixel size. The large (20Mb each) image files were analyzed by the use of Quantity One software (Bio-Rad Laboratories, Hercules, CA) based on the spot coordinates. Each gene has two corresponding spots and the pixel density (intensity) of each spot representing each gene in the array was measured and corrected for the background. The average signal of the pair of duplicate spots were subsequently determined and normalized for comparative studies. To standardize the data from experiments the average intensity for each spot was expressed as a percentage of the total of intensities for all of the spots on the DNA array.

### **Bacterial Growth Conditions for qRT-PCR Experiments**

Overnight cultures were grown in E medium (with appropriate antibiotics) and subcultured the following day in 5 mL fresh E medium with an initial OD of 0.05. Cultures were allowed to grow to an OD of 0.2 before appropriate conditions (control,

75 mM Ca or 10 mM EGTA) were added. The treated cultures were incubated at 37<sup>0</sup>C for approximately 1 hour. Once the cultures reached an OD of 0.4 to 0.5, pellets were obtained by centrifugation at 16,000 x g for 1 min, frozen immediately in dry ice/95% EtOH and stored at -80<sup>0</sup> C freezer.

### **RNA extraction**

RNA was isolated using Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA) as per manufacturer's protocol. RNA was eluted from RNeasy columns 2 times with 30 µL of RNase-free water. Subsequent to RNA extraction, an aliquot of 10 µg RNA for each sample was DNase treated and the DNase was heat-inactivated as per the protocol of New England Biolabs (NEB, Ipswich, MA). RNA concentrations were quantified using a NanoDrop 2000 (NanoDrop Products, Wilmington, DE). The absence of DNA contamination in extracted RNA was confirmed by PCR reaction and subsequent gel electrophoresis.

### **Reverse Transcriptase Reaction and cDNA Synthesis**

GoScript™ Reverse Transcription System (Promega Corporation, WI, USA) was used to reverse transcribe RNA templates. Briefly, for every 20 µL reaction, approximately 5 µg of RNA and 0.5µg random primers (Promega) were incubated at 70<sup>0</sup>C for 5 minutes, chilled on ice for 5 minutes, and then centrifuged briefly before reverse transcription mix (buffer, 3 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, 1 µL reverse transcriptase, water) was added. Annealing at 25<sup>0</sup>C (5 min), followed by extension at

42<sup>0</sup>C (1 hr), and finally inactivation of reverse transcriptase was carried out at 70<sup>0</sup>C for 15 minutes. The cDNA thus prepared was quantified by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware) and diluted to 10 ng/μL working stock in RNase-free H<sub>2</sub>O.

### **qPCR/Real-Time PCR**

Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, California) was used for all real-time qPCR reactions to quantify gene expression levels. Each reaction was performed in a total volume of 25 μL and contained 5 μL of cDNA (50 ng), 12.5 μL SYBR Green (final concentration 1X), 3.75 μL of forward primer (final concentration 120nM), and 3.75 μL of reverse primer (final concentration 120nM). Every sample (housekeeping, controls, unknowns) was represented in triplicate and amplification was performed in a Stratagene Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA). The parameters were as follows: (1) 95°C 10 min, (2) 40 cycles of 95°C for 15 s and 60°C for 30 s, (3) 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s.

Gene-specific primers were designed using PrimerQuest (Integrated DNA Technologies, Inc., Coralville, Iowa) and are listed in Table 3. The primers were designed to ensure optimum amplification of 100 bp to 150 bp of target gene sequence with a primer T<sub>m</sub> of approximately 60<sup>0</sup>C. The specificity of each primer set (used for the amplification of target genes) was verified by analyzing the dissociation curves that were generated with end products of each qRT-PCR reaction. The efficiency of each primer set



was determined using standard curves generated by the MxPro software included with the Stratagene qRT-PCR machine (Agilent Technologies, Santa Clara, CA). While the MxPro software automatically set the threshold value for fluorescence, in some cases the threshold values had to be set manually. The threshold cycle (C<sub>t</sub>) represented the number of reaction cycles required for fluorescence of SYBR Green (individual reaction) to exceed the threshold value. Relative gene expression (fold change) was analyzed by the Pfaffl method (Pfaffl, M.W., 2001), which allowed for the incorporation of individual primer efficiency values in the calculation of fold change ratios.

The Pfaffl formula employed for relative gene expression studies is represented below:

$$R = E_{\text{target}}^{\Delta C_t \text{ target (calibrator – treated)}} / E_{\text{reference}}^{\Delta C_t \text{ reference (calibrator – treated)}}$$

Where,

**E<sub>target</sub>** = amplification efficiency of the target gene

**E<sub>reference</sub>** = amplification efficiency of the reference (housekeeping) gene

**ΔC<sub>t</sub> reference (calibrator – treated)** = the C<sub>t</sub> of the reference (housekeeping) gene in the calibrator minus the C<sub>t</sub> of the reference (housekeeping) gene in the treated sample.

**ΔC<sub>t</sub> target (calibrator – treated)** = the C<sub>t</sub> of the target gene in the calibrator minus the C<sub>t</sub> of the target gene in the treated sample.

Table 3. Primers for qRT-PCR used in the study.

Primer	Primer sequence (5' - 3')	Gene	Reference
arcA-F	CAGATTCATGGTACGGGACAG	arcA	This Study
arcA-R	TGCGTTGATGTTTCCTGACTG	arcA	This Study
rpoS-F	AGCTTATGGGACAACTCACG	rpoS	This Study
rpoS-R	TCTCAACATACGCAACCTGG	rpoS	This Study
rpoB-F	GGTTGGTACTGGTATGGAACG	rpoB	This Study
rpoB-R	AGAACGGGTGTATTTGGTCAG	rpoB	This Study
mdh-F	CCATAGACAGGGTTGCAGAC	mdh	This Study
mdh-R	TCCTGGCGTTAGTTTTACCG	mdh	This Study
ftsZ-F	AGCGAACGATGTACTGAAAGG	ftsZ	This Study
ftsZ-R	GTAGCCCATCTCAGACATTACG	ftsZ	This Study
atoA-F	AAGAGCTTCGTGATGGTGAC	atoA	This Study
atoA-R	GCAGAGTGATATGAATACCCTCC	atoA	This Study
atpD-F	TTTTACCTACACCCGCACC	atpD	This Study
atpD-R	TGTCAAACCTCTCAGGAAGTGC	atpD	This Study
pstS-F	TTACCATCCAGCACCAGTTC	pstS	This Study
pstS-R	CGTTGATTTTGGTGCCTCTG	pstS	This Study
marB-F	GCTGCGCTTATTCTCTTTTCC	marB	This Study
marB-R	TTATCACTGCCAGTACCCATG	marB	This Study
fadB-F	TGTTCGGTTTCTGCCAGTAC	fadB	This Study
fadB-R	ATCTCCACAATCCACCCAAC	fadB	This Study
aas-F	AGATCCATACTTTGTCCGCAG	aas	This Study
aas-R	CAAAACCATCTTCACTTCCCG	aas	This Study
cheB-F	TGACTTCTGACCCTTTGCC	cheB	This Study
cheB-R	CTGACGCTGGATGTTGAAATG	cheB	This Study
ybbO-F	CTGACTTTAATTCCGCTGTGG	ybbO (b0493)	This Study
ybbO-R	GGTGAAGGGCGTATTGTGATG	ybbO (b0493)	This Study
ycfL-F	GCATCCTCAACGCTTTATAACG	ycfL (b1104)	This Study
ycfL-R	GAATGTGCGGGAATGGTAAC	ycfL (b1104)	This Study
yfaD-F	GCCGTTGCTGTTTTATCATGG	yfaD (b2244)	This Study
yfaD-R	ATCGTCAAACCTCATCCAGCC	yfaD (b2244)	This Study
corA-F	CGGAAGTGAAGACATCGAAG	corA	This Study
corA-R	AGAAGGAGTGAATATGCAGGC	corA	This Study
secA-F	CAAACGAACTGACCAAAGCC	secA	This Study
secA-R	CAATATCTGTACCACGACCCG	secA	This Study
yjeE-F	CACTTTGATTTGTACCGCCTTG	yjeE (b4168)	This Study
yjeE-R	TGTGTATTTTCGACATCCGGG	yjeE (b4168)	This Study
pqiB-F	GATCGCTGAAAACCGGAAAC	pqiB	This Study
pqiB-R	GCTAACGGTCGGGATAATCTG	pqiB	This Study

## CHAPTER 3

### RESULTS AND DISCUSSION

#### **Isolation of Calcium-Sensitive Mutants of *E. coli* by *TnphoA* Mutagenesis**

The 7.7 kb transposon *TnphoA* contains Kan<sup>r</sup> gene as a selective marker and the reporter gene for alkaline phosphatase *phoA* (Figure 1). Following transposon mutagenesis, penicillin enrichment was used to aid in the isolation of calcium-sensitive mutants. Several new calcium-sensitive mutants were identified by this transposon mutagenesis procedure. The calcium-sensitive mutants thus generated are described in Table 1 and photomicrographs of some mutants are included in Figure 2. The mutants fell into three general classes: (i) Ca<sup>2+</sup>-sensitive chemotaxis mutants, (ii) Ca<sup>2+</sup>-sensitive cell division mutants, and (iii) Ca<sup>2+</sup>-sensitive mutants that showed no defects in cell division or chemotaxis (Table 2). While some of the mutants were sensitive to 50 mM CaCl<sub>2</sub>, sensitivity measured by growth inhibition assay revealed the inability of most mutants to grow in the presence of 75 mM CaCl<sub>2</sub> on plates and in broth cultures.

Calcium-sensitive mutants were generated by use of the transposon, *TnphoA*. The reporter gene for alkaline phosphatase *phoA* has no promoter or Shine Dalgarno sequences, and is only expressed when the fusion is in-frame. When inserted in frame and in proper orientation, *TnphoA* will randomly mutagenize bacterial chromosome by disrupting the gene the transposon will insert itself in. In addition, *TnphoA* will fuse alkaline phosphatase to the amino terminal of the protein product of the disrupted gene.

Alkaline phosphatase is functional when it is fused to a signal that promotes the export of the protein to the envelope of the cell (such as periplasmic, outer membrane or cytoplasmic membrane proteins). Our search for calcium sensitive *E. coli* mutants was thus targeted to outer membrane protein genes by essentially isolating *phoA* gene fusions. Infection of a *phoA* deletion strain CC118 with  $\lambda$ Tn*phoA*, followed by penicillin enrichment and subsequent screening of blueness of colonies on XP resulted in mutants with Tn*phoA* insertions exhibiting PhoA activity.

### **Physiological and Biochemical Properties of Calcium-Sensitive Mutants**

The cell division mutants (e.g., Cal134) were easily identified microscopically as long “snake-like” cells (Figure 2). One of the  $\text{Ca}^{2+}$ -sensitive cell division mutants (Cal134) grew as a long filamentous cell that was over 20 cell-body length in size. Multiple chromosomes within these snake-like cells were observed following DAPI staining of this mutant (Figure 3). These results imply that this mutant was defective in cell division, but not DNA replication.

The generation of these  $\text{Ca}^{2+}$ -sensitive cell division mutants was not unexpected. Holland and co-workers (Bouquin and Seror, 1995; Casarégola and Holland, 1991) isolated mutants that were resistant to different calcium antagonists and are defective in cell division. They proposed a model suggesting that calcium plays a role in the control of bacterial growth and its cell cycle (Holland *et al*, 1990; Norris *et al*, 1988).

The sensitivity of these calcium-sensitive mutants to other cations was tested and their minimal inhibitory concentration (MIC) values for these cations are shown in

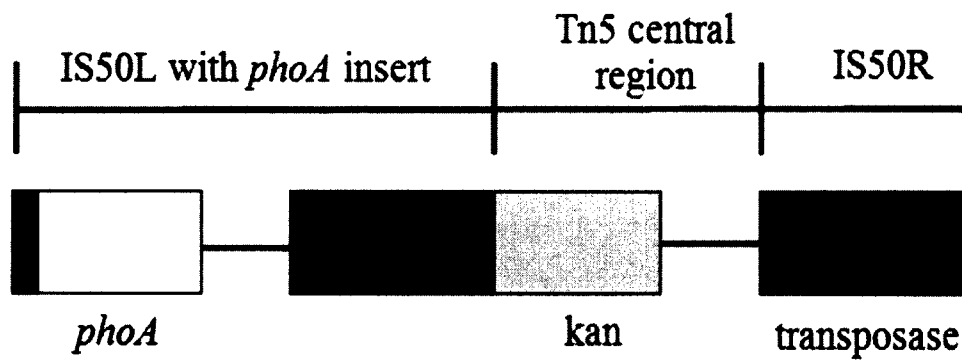


Figure 1. The transposon Tnp<sub>phoA</sub> (Manoil and Beckwith, 1985). The 7.7 Kbp transposon is derived from Tn5 with the selective marker Kan and the reporter gene *phoA*. Successful (in frame) gene-transposon fusion allows for identification of secreted and transmembrane proteins.

Figures 5 to 7. Besides calcium-sensitivity, all of the mutants exhibited increased sensitivities to several divalent cations ( $\text{Ni}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Cd}^{2+}$ ). Mutant Cal117 was sensitive to all of the cations tested. Mutants Cal134 and Pho43 exhibited sensitivity to  $\text{Co}^{2+}$  and  $\text{Zn}^{2+}$ , while Cal134 was also sensitive to  $\text{Cu}^{2+}$ . Cal102 was sensitive to all of the cations except  $\text{Ni}^{2+}$ , and Cal512 exhibited a similar pattern of sensitivity as Cal117 though it was not sensitive to  $\text{Mn}^{2+}$ . It is noteworthy to mention that all calcium-sensitive mutants were resistant to  $\text{Mg}^{2+}$  (Figure 7).

In other ion transport systems, mutants defective in ion transport show increased sensitivity to diverse additional ions.  $\text{Na}^+$  transport mutants are resistant to  $\text{Li}^+$  (Park and Lusk, 1976), while  $\text{Mg}^{2+}$  transport (*corA*) mutants are resistant to  $\text{Co}^{2+}$  and sensitive to  $\text{Ca}^{2+}$  (Maguire, 1993). We expected that the *cal* mutants isolated above would show sensitivities to other cations or that they would show an increased resistance to some cations.

Alkaline phosphatase activities for *TnphoA* calcium-sensitive mutants were assessed using plate assay that contained 5-bromo-4-chloro-3-indolyl phosphate (XP). XP is a substrate for alkaline phosphatase. The blueness of colonies indicated the generation of successful calcium-sensitive mutants that had gene fusion products that are exported out of the cytoplasm or located in periplasmic face of membrane proteins. In addition to turning blue on E medium plates with XP, the effect of culture conditions on alkaline phosphatase activity for the mutant strain Pho43 is shown in Figure 4. In addition, alkaline phosphatase activity was assayed for Pho43 at different time intervals

Table 4 summarizes the physiological and biochemical characterization data for a comprehensive representation of observations made on the transposon mutants.

### **Molecular Characterization of Calcium-Sensitive Mutants**

The sites of the gene fusions for three mutants were identified by arbitrarily-primed PCR (arb-PCR). Since the PCR products of arb-PCR contained part of the upstream regions of the transposon *TnphoA* fused to the disrupted gene, the start of *cal* gene was easily recognized. The resulting partial gene sequence was used to search the compiled DNA sequence databank of *E. coli* genome by the use of the BLAST program at NCBI. The sites of the gene fusion for three *cal* mutants were at the *fdoG*, *gpt* and *pqi* genes (Table 5). The *fdoG* gene codes for the  $\alpha$ -subunit of formate dehydrogenase (Wang and Gunsalus, 2003), while the *gpt* gene codes for guanine-xanthine phosphoribosyltransferase phosphotransferase (Pratt and Subramani, 1983) and *pqi* gene is induced by paraquat and regulated by SoxRS (Koh and Roe, 1995). The arbitrarily-primed PCR fragment of Cal134 exhibited 96% sequence homology with *fdoG* gene and also a significant match as evidenced by the e value of 5e-12. Similarly, the *cal* genes of PhoC23 and PhoA4 showed significant matches to genes *gpt* and *pqiB* (e value of 2e-44 and 1e-25, respectively) with 98% sequence identity for both. Primers *TnphoA*-F and *TnphoA*-R were used to amplify a 3 kb segment of the transposon *TnphoA* (Table 2). The presence of the transposon *TnphoA* in the mutant cells was confirmed as evidenced in figures 8 and 9. In Figure 8, lanes 5, 6, and 8 represent the transposon mutants PhoC23, PhoA4, and Cal117, respectively. The presence of a 3Kb band also confirmed the

presence of *TnphoA* within these mutants. In addition, Figure 9 exhibits the presence of the amplified 3 Kb segment of *TnphoA* in the mutant strain Cal134 (lane 5), thus confirming the incorporation of the transposon in these calcium-sensitive mutants.

The site of the *TnphoA* insertion was detected by hybridization of a DIG-labeled *TnphoA* derived probe to membrane blotted nucleic acids. The 162 base pair probe DNA was labeled with digoxigenin by PCR. The hybridized probes were immunodetected with anti-DIG-alkaline phosphatase and then visualized with the chemiluminescent substrate CSPD as detailed in the Boehringer Mannheim kit. Hybridization experiments using this *TnphoA* derived probe confirmed that each mutation was distinct and the result of the single insertion of *TnphoA* (data not shown).

The sites of the gene fusion for three cal mutants were the following: gene (Cal134), *gpt* gene (PhoC23), *pqi* gene (PhoA4). Our initial observations with the *TnphoA*-generated cal mutants suggest that their calcium-sensitivity was the result of a number of diverse genes being disrupted by the insertion of the transposon. These results suggest a pleotropic nature for the cal mutations and indicate that many genes may be regulated by calcium. Because of the results from these experiments, we decided to radically change our approach. We wanted to investigate global gene expression under growth conditions with and without calcium with a broader goal of identifying all of the calcium-regulated genes in *E. coli*.



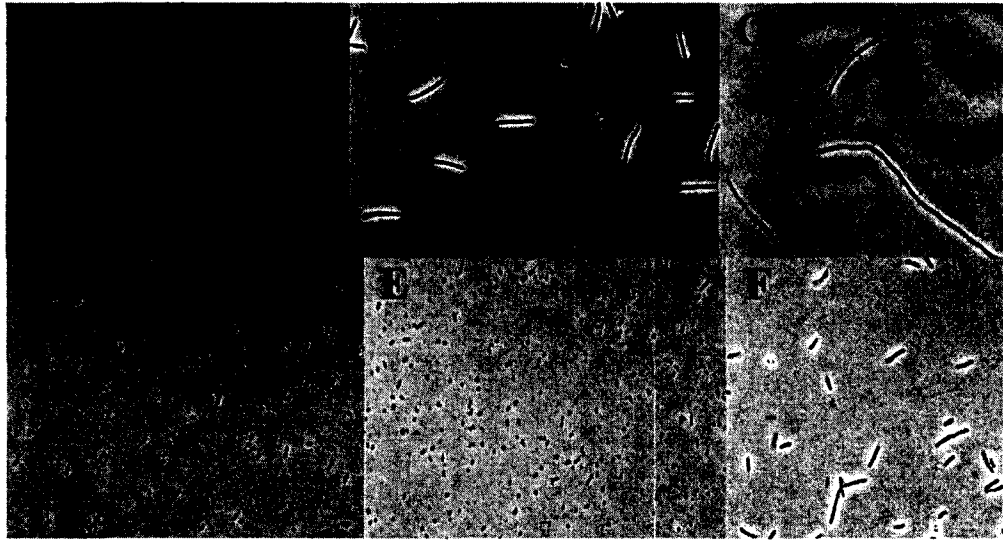


Figure 2. Photomicrograph of wild type *E. coli* and calcium-sensitive mutants. A CC118 with wild type morphology, B Cal102 cells with a snake-like morphology consist of up to 10 times the length of the wild type CC118, C Cal134 cells were more elongated at about 10 to 20 times the length of CC118, D and E Cal 117 and Pho43, respectively, had wild type morphology, and F Pho43 are long like Cal 102.

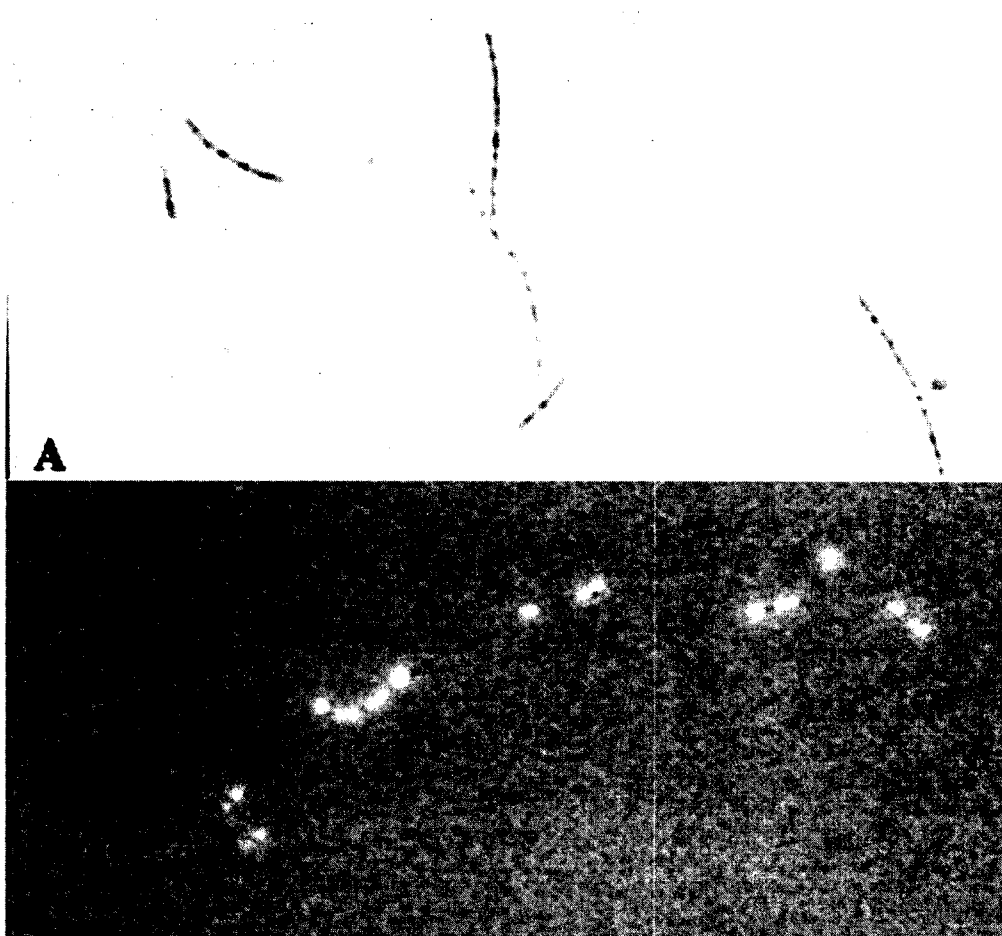


Figure 3. The Cal134 mutant is defective in cell division, but not DNA replication. DAPI stained phase contrast A and fluorescent B pictures of Cal134 long snake cell division mutants.

Table 4. Properties of calcium-sensitive mutants

Strain	Morphology	Motility	Swim Rate mm per 8 hours	Alkaline Phosphatase (plate assay)
CC118 (WT)	Short Rods	Smooth-Tumbly	49 mm	-
Cal117	Medium Snakes	Smooth	0 mm	++
Cal512	Medium Snakes	Smooth	2 mm	+
Cal102	Medium Snakes	Tumbly	14 mm	++
Pho43	"Sausage" Snakes	Nonmotile	0 mm	++
Cal134	Long Snakes	Smooth	4 mm	++
PhoA4	Short Rods	Smooth-Tumbly	37 mm	+++
PhoC23	Short Rods	Tumbly	23 mm	-
Cal 719	Medium Snakes	Smooth-Tumbly	31 mm	ND
Cal526	ND	ND	11 mm	ND
Cal504	ND	ND	12 mm	ND

Medium = 5-8 cell lengths, Long = 10-20 cell lengths

- = no color + = light blue ++ = medium blue +++ = dark blue

ND = not determined

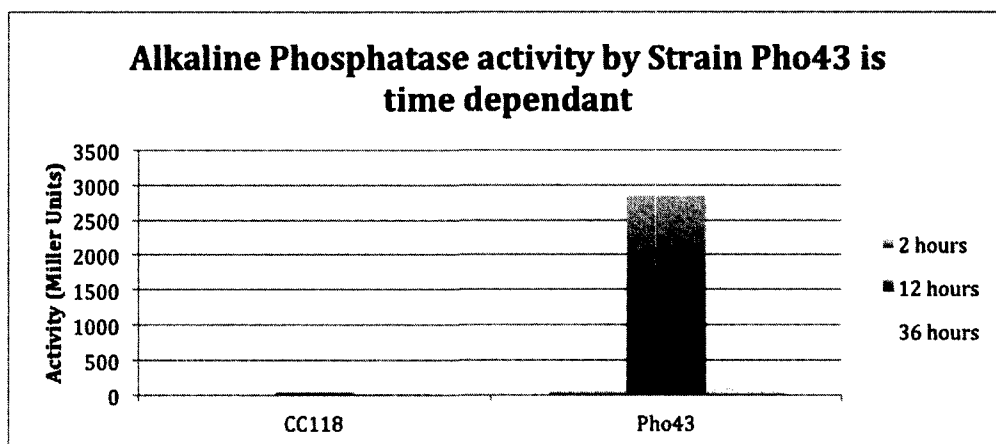


Figure 4. Alkaline Phosphatase activity by Pho43 is time dependent. At different time intervals alkaline phosphatase activity was assayed as described in the method.

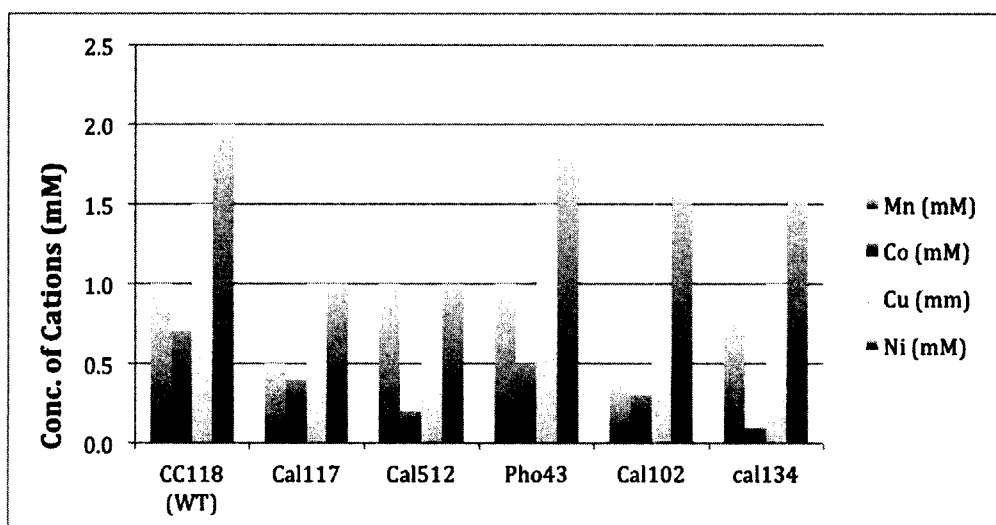


Figure 5. Ca-sensitive mutants were sensitive to other divalent cations. MIC values for cations tested were determined as described in the Methods. Mutants Cal117 and Cal512 exhibit a similar pattern of cation sensitivity, while Cal102 and Cal134 exhibit similar profiles. The wild type CC118 and mutant Pho43 have identical MICs for  $\text{Mn}^{2+}$  and  $\text{Cu}^{2+}$ .

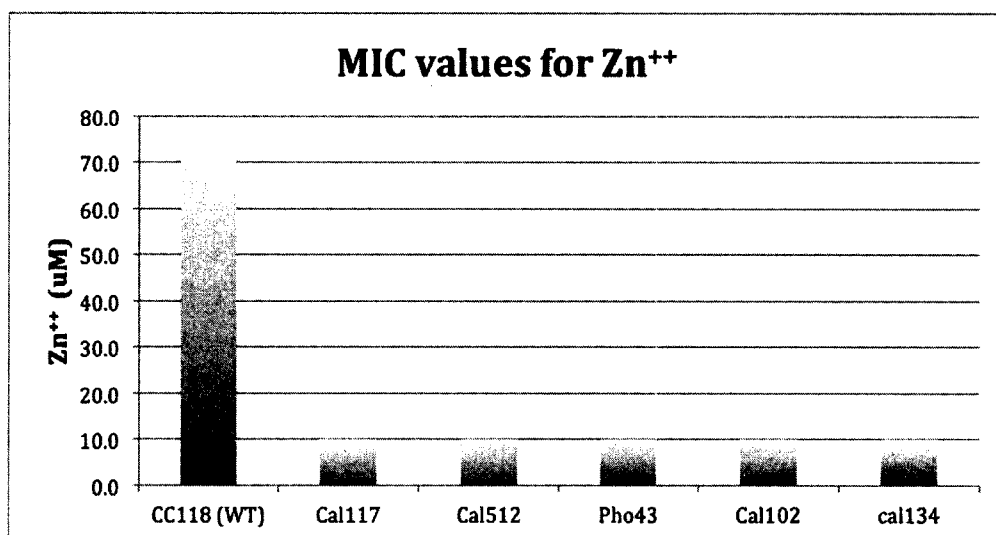


Figure 6. MIC values for Zn in the Ca-sensitive mutants

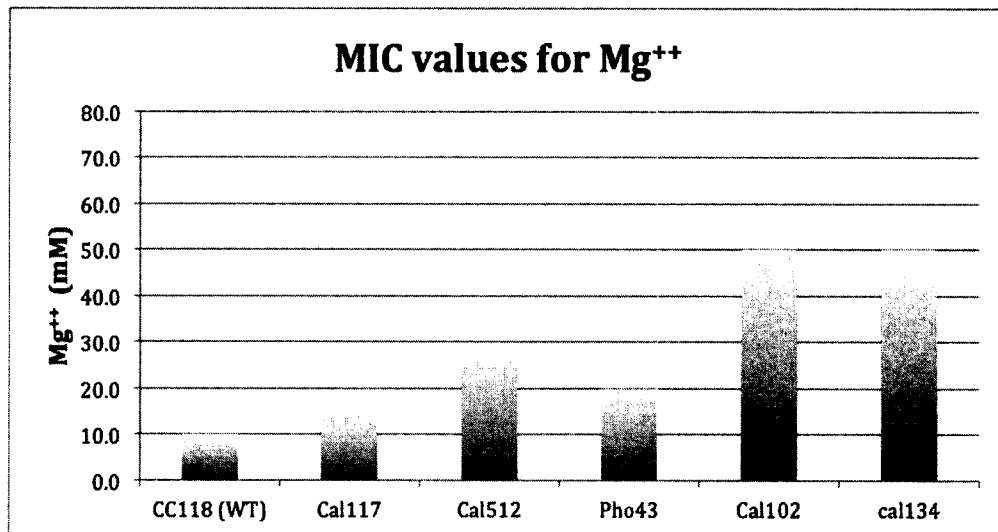


Figure 7. MIC values for Mg<sup>2+</sup> in the Ca-sensitive mutants

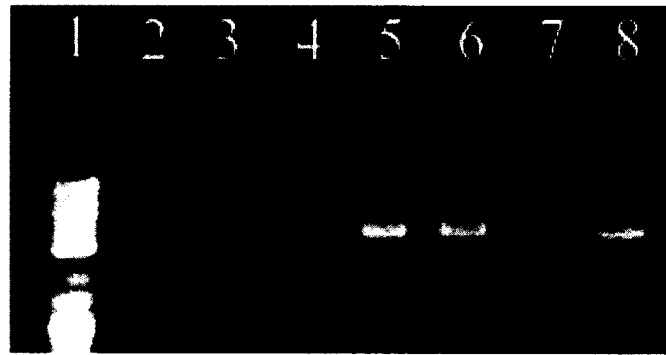


Figure 8. PCR amplification of transposon TnphoA. Lanes 5, 6, and 8 represent the mutants PhoC23, PhoA4, and Cal117, respectively. The visible bands at 3Kb in the mutants indicate the presence of TnphoA. Lane 1 was loaded with 2-log DNA ladder (NEB) and lane 3 had wild type amplified CC118 DNA exhibiting the absence of transposon.



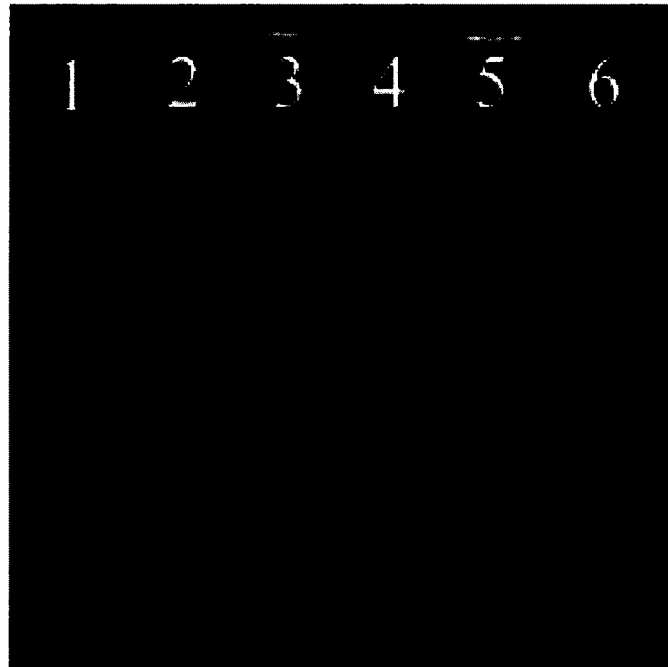


Figure 9. PCR amplification of transposon *TnphoA* in Cal134. Lane 4 and 5 represent PhoC23 and Cal134 and indicate the presence of *TnphoA*. Lane 1 was loaded with 2-log DNA ladder (NEB) and lane 2 had wild type amplified CC118 DNA exhibiting the absence of transposon.

Table 5. *cal* gene mutations identified by ARB-PCR

<b>Mutant</b>	<b><i>cal</i> gene</b>	<b>Gene function</b>	<b>e-value</b>	<b>% identity</b>
Cal134	<i>fdoG</i>	Formate dehydrogenase (Allows the use of formate as major electron donor during aerobic respiration)	5e-12	96%
PhoC23	<i>gpt</i>	guanine-hypoxanthine phosphoribosyltransferase (involved in salvage of nucleosides and nucleotides)	2e-44	98%
PhoA4	<i>pqiB</i>	paraquat-inducible protein (inducible by superoxide generating radical paraquat and regulated by SoxRS)	-	98

### **Panorama *E. coli* Gene Array**

With the availability of nylon membrane arrays (specifically Panorama gene arrays), we decided to investigate global gene expression in wild-type cells, which maintain a steady-state level of cytoplasmic calcium. Our goal was to first assess the transcriptome profile of *E. coli* MG1655 and classify genes into functional categories. Armed with such structured information, we would then focus our attention to mutants that did not regulate cytoplasmic  $\text{Ca}^{2+}$ -levels (e.g., *calC* and *calD*). In the presence of elevated calcium, their cytoplasmic  $\text{Ca}^{2+}$ -levels increase, while in the presence of EGTA, their cytoplasmic  $\text{Ca}^{2+}$ -levels decrease. Since the cytoplasmic calcium level in these mutants is not regulated, experiments with elevated calcium (75mM Ca) or depleted calcium (10mM EGTA) would measure the effect of cytoplasmic calcium on global gene expression. My efforts only addressed the wild-type MG1655 global profile using elevated and depleted calcium conditions. Later I focused on more quantitative analysis of specific genes to elucidate their possible involvement in calcium transport and homeostasis.

Panorama *E. coli* DNA macroarrays (Sigma\_Genosys) representing all 4290 protein-coding genes were used to identify those genes that are globally regulated by calcium and to observe the effects of elevated and depleted calcium levels on the global transcription profile (Figure 11).

It was crucial to check both the integrity of the RNA and the amount of genomic DNA contamination by 1.2% agarose gel electrophoresis. The 23S and 16S ribosomal

RNA bands were clearly visible at about 2:1 ratio (23S:16S) of staining intensity. Genomic DNA contamination in the RNA sample is usually seen as high molecular weight-staining material. The processed RNA samples were devoid of any such contamination as evidenced by agarose gel electrophoresis.

The corresponding spots from samples on arrays were compared to identify the fold-induction (upregulation) or fold-reduction (downregulation) in expression between the samples (Figure 11). By this method of array analysis a 2-fold difference in expression was considered as important. Changes in expression (fold change) of genes during elevated and depleted calcium conditions are shown in Table 6.

Previously *E. coli* mutants were demonstrated to have calcium-regulated polypeptide synthesis (Laoudj *et al*, 1994). *E.coli* mutants resistant to calcium channel inhibitors, such as verapamil and diltiazem, have slightly elevated cytoplasmic free-Ca<sup>2+</sup> levels. In their study, Laoudj *et al* (1994) used these mutants, grew in minimal medium at 37° C with aeration to late exponential phase of OD<sub>600</sub> of 0.6. They identified the induction of at least 25 polypeptides when cells were treated with calcium chelators such as EGTA and BAPTA. In addition, they saw the repression of at least 11 polypeptides using 1-D and 2-D gel electrophoresis. Three of the induced major polypeptides cross-reacted with polyclonal and monoclonal antibodies to calmodulin or calerythrin.

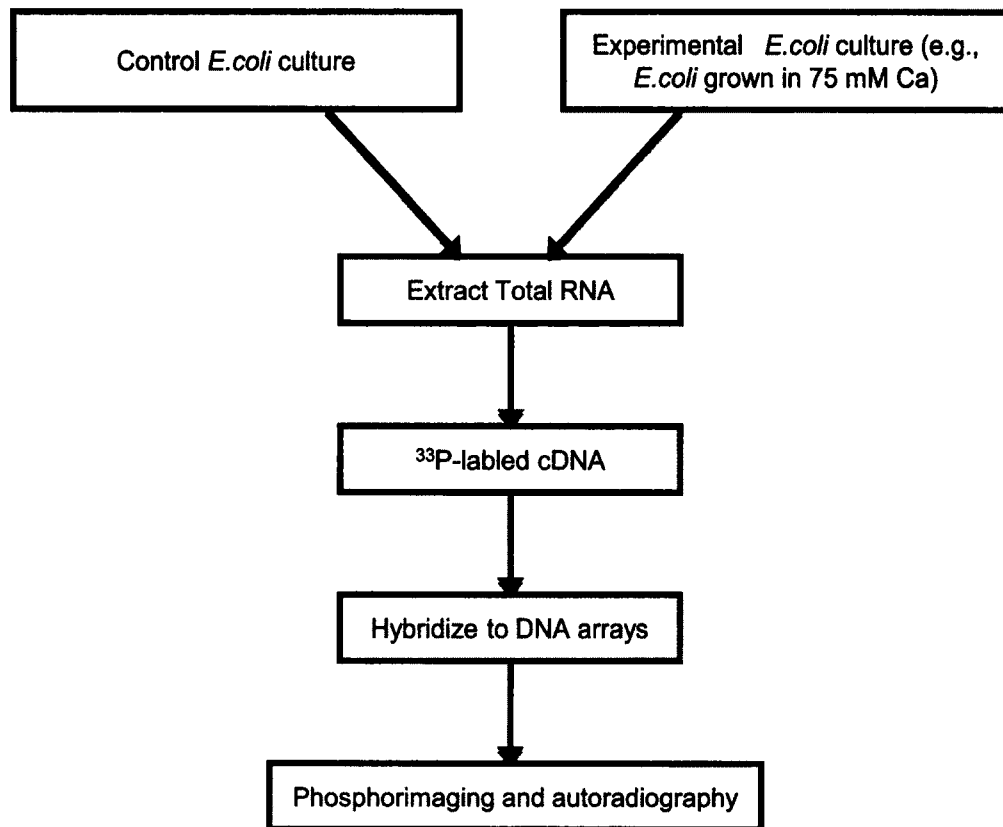


Figure 10. *E.Coli* DNA Macroarray Protocol. Cultures were grown under specific conditions for comparing gene expression. The total RNA from each culture was then extracted intact and free from any contaminating gDNA. Radioactively labeled cDNA was generated by reverse transcription. The labeled cDNA was hybridized to duplicate macroarrays.

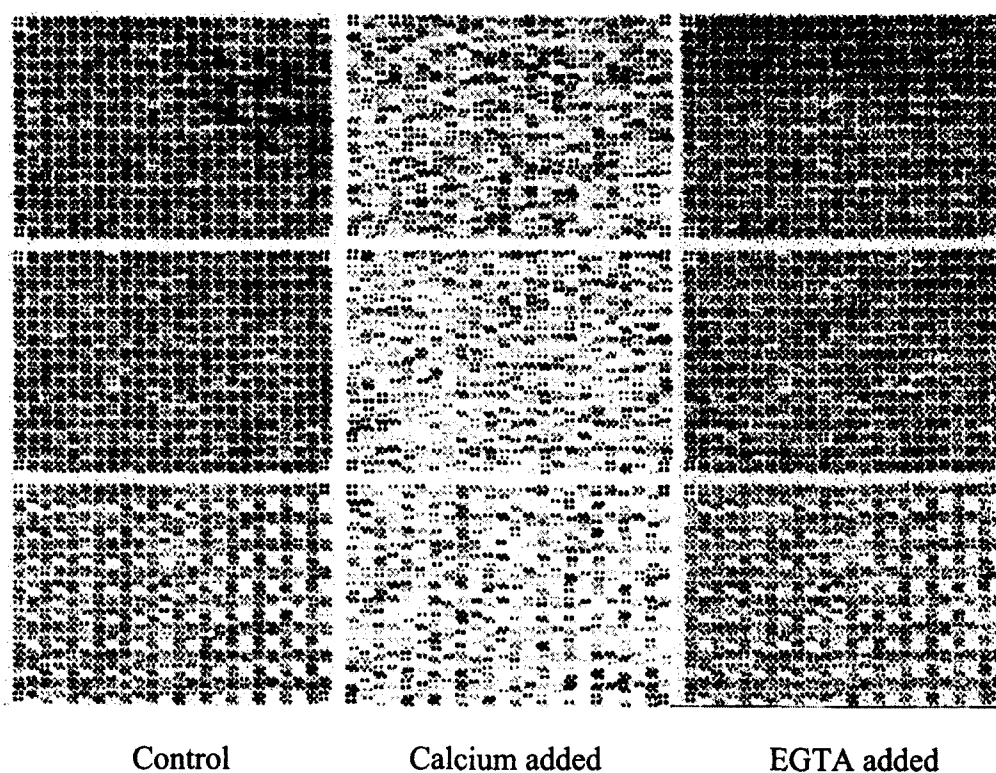


Figure 11. Panorama *E. coli* gene array exhibiting global gene expression profiles of *E. coli* MG1655 following growth on E medium (control), E medium supplemented with 75mM calcium, and E medium supplemented with 10mM EGTA.

Table 6. Panorama macroarray gene expression profiles and fold change as compared to control condition. The table shows log2 (expression ratio) values of the genes under different conditions. Values greater than 0 means upregulation, while values less than 0 means downregulation.

Gene	Gene function	Calcium Treat (fold change)	EGTA Treat (fold change)
cheB	Protein-glutamate methylesterase	-0.3	0.4
pstS	Periplasmic phosphate-binding protein	0.9	-0.2
ftsZ	Cell division protein FtsZ	-0.2	-1.7
marB	Multiple antibiotic resistance protein MarB	-0.3	-0.5
aas	Fatty acid and phosphatidic acid biosynthesis	1.5	-1.7
fabI	Enoyl-[acyl-carrier-protein] reductase (NADH)	0.7	-0.5
fadB	Degradation of small molecules: Fatty acids	-0.3	-1.3
gyrA	DNA gyrase subunit A	2.4	-2.3
pqiB	Not classified	-0.5	-1.7
chaA	Putative calcium/proton antiporter	-0.3	-2.3
chaB	Cation transport regulator ChaB	-1.0	1.1
B1428	Unknown	-2.3	2.3

### **qRT-PCR and Comparative Analysis of Expression of Genes of Interest**

Quantitative reverse transcriptase PCR (qRT-PCR) was used to measure individual gene expression (treated with either calcium or EGTA). For each experimental condition, the expression levels of gene transcripts are shown as a ratio that is relative to expression in the untreated control samples.

Some of the common genes included in both Panorama array experiments and qRT-PCR experiments enabled me to compare the expression profiles between the two different approaches used to assess gene expression levels in MG1655 strains under control, calcium-treated and EGTA-treated conditions. For *cheB* gene, a downregulation was observed in the presence of calcium during array and qRT-PCR analysis, however, array results indicated an upregulation (Table 6) of the gene under EGTA treatment while qRT-PCR indicated downregulation (Figure 12). As witnessed in qRT-PCR, addition of 75mM calcium (elevated calcium condition) downregulated *cheB* almost 10-fold, while treatment with 10 mM EGTA (depleted condition) downregulated *cheB* expression 5–fold (Figure 12).

For gene *pstS*, under calcium addition, array results exhibited a two-fold increase ( $\log_2$  of 0.9) while qRT-PCR results revealed no change as compared to control condition (Figure 12). Under calcium-depleted condition, the expression levels were downregulated in both procedures. *ftsZ*, *marB*, *fadB* and *pqiB* genes were downregulated under both elevated and depleted calcium conditions in both methods. However, the exact level or fold of relative expression changes may not be identical between the arrays and qRT-



PCR procedures. The fundamental difference between global expression analysis using arrays and individual gene expression measured by qRT-PCR procedure is that qRT-PCR places a more individualized quantitative emphasis. As such, array results are usually further verified and validated by qRT-PCR. I had the opportunity to use the Panorama *E. coli* arrays to assess the global transcriptome profile, however, I was unable to use multiple replicates due to resource constraints and my efforts were limited by the availability of Panorama arrays. The results of qRT-PCR therefore were not expected to corroborate all the array results and this has been observed when the results were compiled and compared as evidenced in Figure 12 and Table 6.

### **15 Genes of Interest**

Comparative analysis of gene expression as quantified by qRT-PCR experiments centered on 15 genes that were selected for their purported involvement in calcium homeostasis based on published data in the recent literature (Naseem *et al*, 2009; Theodorou and Kyriakidis, 2009) and my preliminary global expression data from Panorama macroarray. Of the 15 genes chosen for this study, 8 genes (*cheB*, *pstS*, *ftsZ*, *marB*, *fadB*, *pqiB*, *ybbO* and *yfaD*) were initially included in the global expression profiling of wild type *E. coli* MG 1655 grown in control, elevated and depleted calcium conditions during Panorama DNA macroarray experiments. Of the remaining 7 genes, 3 genes (*atpD*, *secA*, and *yjeE*) were selected based on the calcium efflux observations made by Naseem *et al* (2009). The gene *atoA* was selected due to its implication in calcium-regulation of cPHB [complexed poly-(R)-3-hydroxybutyrate] biosynthesis in *E.*

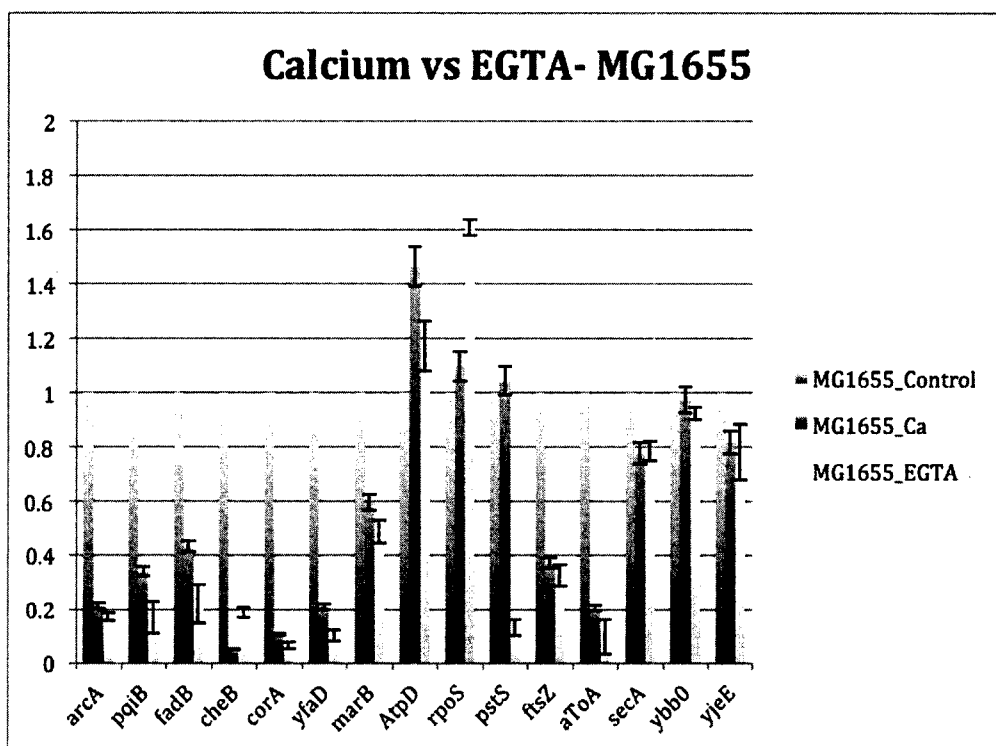


Figure 12. Relative expression of 15 genes under elevated  $\text{Ca}^{2+}$  condition (75mM Ca) and depleted  $\text{Ca}^{2+}$  condition (10mM EGTA) in MG1655. MG1655 (control) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer.

*coli* (Theodorou and Kyriakidis, 2009). The gene *corA* was included because of previously published results indicating that *corA* mutants were defective in magnesium transport system and were sensitive to calcium (Park et al., 1976). Brey and Rosen (1979) also showed that *corA* mutants were sensitive to 50 mM calcium. The *arcA* and *rpoS* genes were chosen to assess general response to stress in calcium-sensitive mutants.

### **Relative Expression of Parental and Mutant Strains under Control Condition**

Gene expression of the mutants and their counterpart parental strains grown in the presence of 75 mM calcium (elevated calcium condition) and in the presence of the chelator 10 mM EGTA (depleted calcium condition) was determined. To be prudent these data were analyzed to ascertain the fold change ratio between parental wild type and their corresponding mutants under control (untreated; no added  $\text{Ca}^{2+}$  or EGTA) conditions to establish a baseline. The data indicate that the expression of 15 selected genes in all of parent/corresponding mutants sets under control conditions were very similar to one another (Figures 13 and 14).

### **Relative Expression of 3 Parental Wild Type Strains under Control Condition**

*E. coli* MG1655 was used during the Panorama DNA macroarray experiment. To corroborate and validate the preliminary array results, I included strain MG1655 in qRT-PCR experiments concomitant with the investigation of calcium-sensitive mutants and their parental counterparts. Inclusion of MG1655 strain was advantageous because of the following reasons: (i) the readily available sequence information and genome databases,

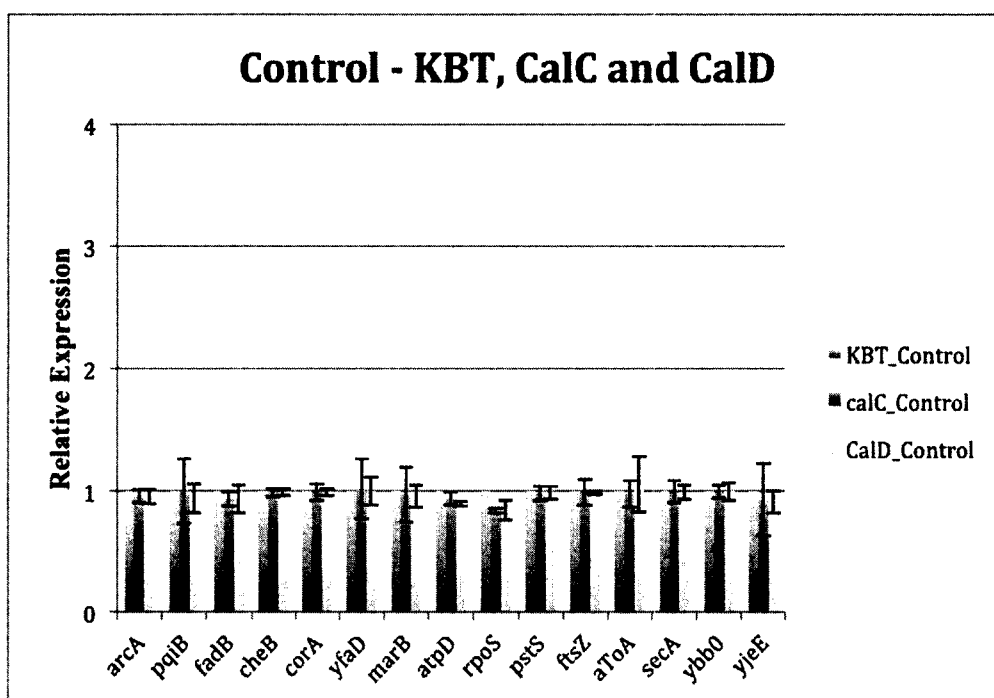


Figure 13. Relative expression of parental (KBT) versus mutant strains (calC and calD). Under control (untreated) condition, no difference was observed in gene expression between calcium-sensitive mutants and wild type parent. KBT (untreated) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer.

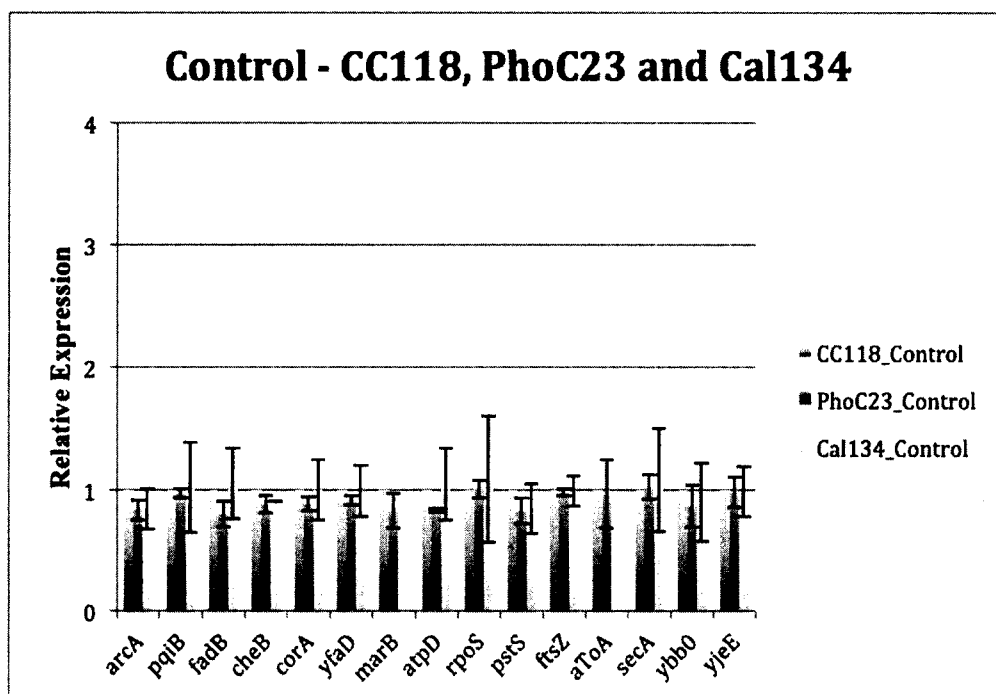


Figure 14. Relative expression of parental (CC118) versus mutant strains (PhoC23 and Cal134). Under control (untreated) condition, no difference was observed in gene expression between calcium-sensitive mutants and wild type parent. CC118 (untreated) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer.

(ii) the preliminary relative expression data obtained from Panorama macroarray, and (iii) the opportunity to look at the big picture which was not just with calcium-sensitive mutants but also to assess expression changes (upregulation/downregulation) in another wild-type strain during control, elevated and depleted calcium conditions. The relative expression of 15 selected genes in the 3 parental wild type strains in the untreated control condition were very similar to one another (Figure 13).

Because there were no differences in relative expression profiles between the mutants and parents under control condition (Figures 13 and 14), any changes in gene expression under elevated or depleted calcium conditions would be expected to reflect a genuine response to such conditions. In addition, a similar pattern of relative expression in all wild type strains (MG1655, KBT, CC118) under control conditions as exhibited in Figure 15 would allow us to look at a more comprehensive profile of gene expression (inclusive of results obtained from array experiments) during calcium homeostasis.

### **Analysis of Individual Gene Expression**

Due to the fact that the 15 genes selected for the qRT-PCR experiment did not easily classify into functional groups and because there were multiple parent/mutants each with its own control, elevated and depleted calcium conditions, I deemed it more relevant to assess relative expression levels of one gene at a time. Such an effort unambiguously focused on the participatory role of each gene in calcium homeostasis in *E. coli*. The rationale was to initially determine global expression profile with Panorama macroarray, to subsequently focus on a subset of genes that can be subjected to

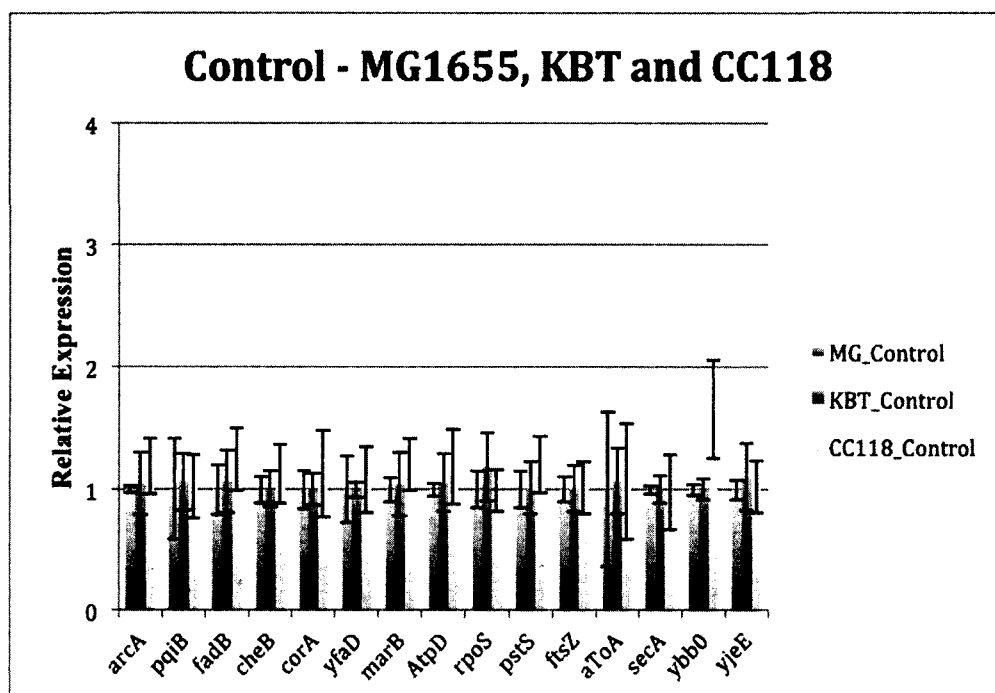


Figure 15. Relative expression of parental wild type strains (MG1655, KBT, CC118). Under control (untreated) condition, no difference was observed in gene expression between MG1655 and the wild type parents. MG1655 (untreated) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer.

quantitative verification by qRT-PCR, and to finally answer the question: what genes are regulated by calcium?

### **atpD Expression**

Naseem *et al* (2009) have recently demonstrated *atpD* (F1 complex,  $\beta$  subunit of ATP synthase) as a potential calcium efflux gene in *E. coli*. The researchers identified a knockout mutant of *atpD* as defective in  $\text{Ca}^{2+}$  efflux by measuring cytosolic calcium levels with the aid of calcium-sensitive luminescence of aequorin. The knockout mutant exhibited lowered ATP levels concomitant with defect in calcium efflux. In their experiment, addition of EGTA to *atpD* knockout cells did not decrease the cytosolic free calcium levels, while EGTA caused a rapid decrease in cytosolic free calcium in wild-type cells.

The CalC and CalD mutants employed in my investigation are defective in  $\text{Ca}^{2+}/\text{PO}_4^{2-}$  symporter activity (Brey and Rosen, 1979). While the wild-type parental cells (KBT001) have a cytosolic free- $\text{Ca}^{2+}$  level of about 105 nM, the free- $\text{Ca}^{2+}$  levels for CalC and CalD are approximately 1130 nM and 410 nM (Tisa and Adler, 1995a). In addition, the mutants are unable to regulate cytoplasmic  $\text{Ca}^{2+}$  levels as evidenced by an increase in calcium during addition of calcium to the growth medium and by a decrease in cytoplasmic  $\text{Ca}^{2+}$  levels when grown in the presence of EGTA.

The qRT-PCR results showed at least a 3-fold upregulation of *atpD* in CalC and CalD mutants during elevated calcium condition (Figure 16). Interestingly, addition of EGTA resulted in a 10-fold upregulation of *atpD* (Figure 17) as would be expected if the



cells are to efflux out calcium while the chelator in the growth medium drives calcium out of the cell and if *atpD* acts as the efflux gene as explained by Naseem *et al* (2009). The Tn*phoA* transposon mutant PhoC23 on the other hand exhibited a slight (approx. 2-fold) downregulation of *atpD* as compared to the parental strain CC118 in both elevated and depleted calcium conditions (Figures 18 and 19). PhoC23 is a tumbly mutant (Table 4). Whether a defective efflux system is contributing to elevated cytoplasmic free calcium in these mutants is an interesting question – one that would require intracellular calcium measurements. The wild-type MG1655 revealed a minor increase in *atpD* expression (approx. 1.2 – 1.4 fold) in both elevated and depleted calcium conditions. One would presume the wild-type cells to have a functional *atpD* regulation to allow for calcium efflux out of the cell as necessary and my results would indicate such a scenario.

### **marB Expression**

The gene *marB* (219 bp) encodes a putative protein of unknown function and is thought to have a role in multiple antibiotic resistance and in the regulation of antibiotic efflux pump *acrAB*.

In the qRT-PCR experiments, the CalC and CalD mutants had 15-fold and 3-fold upregulation of *marB* under elevated and depleted calcium conditions, respectively (Figures 16 and 17). The transposon mutant PhoC23 exhibited a high upregulation of 40-fold in both elevated/depleted calcium conditions (Figures 18 and 19). The wild-type MG1655 strain had a slight downregulation of approx. 2-fold during elevated/depleted

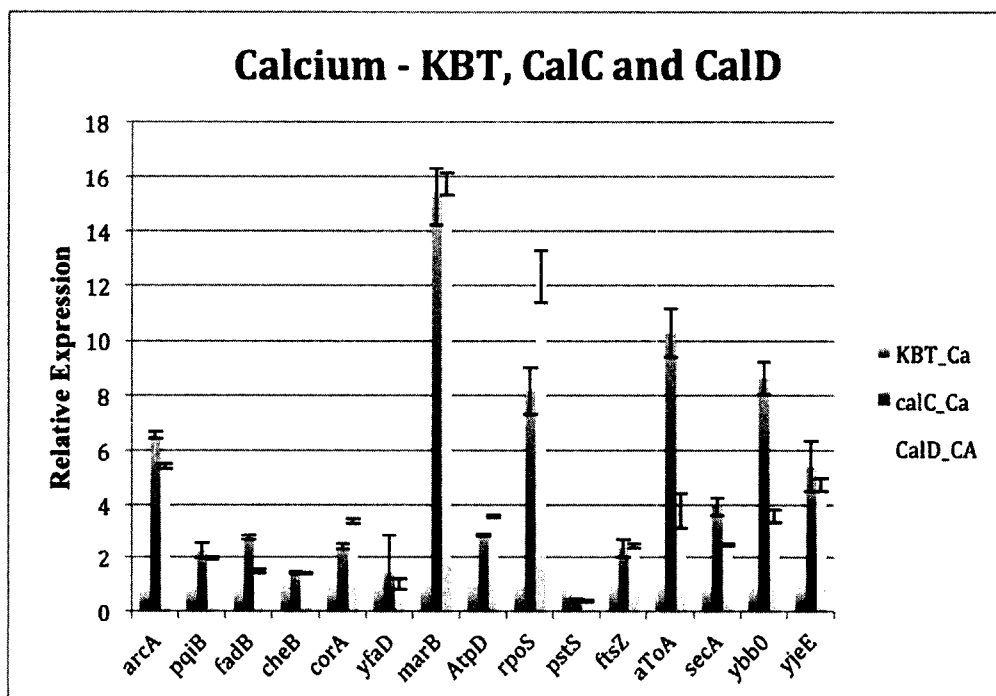


Figure 16. Relative expression of parental (KBT) versus mutant strains (calC and calD) under elevated calcium conditions (75 mM  $\text{Ca}^{2+}$ ). KBT (calcium-treated) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer.

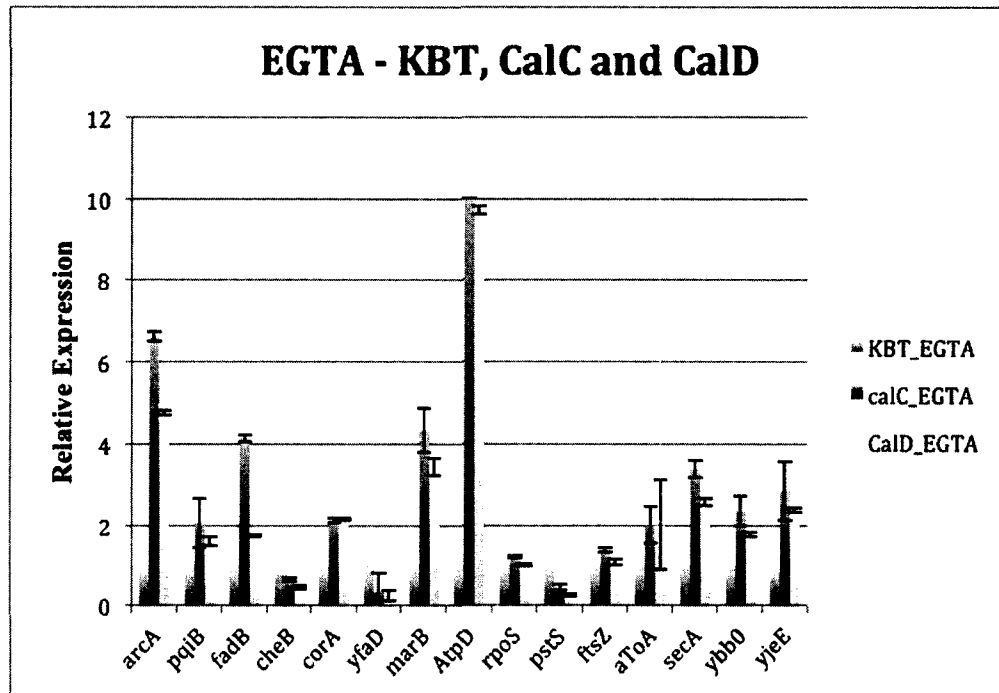


Figure 17. Relative expression of parental (KBT) versus mutant strains (calC and calD) under depleted calcium conditions (10 mM EGTA). KBT (EGTA-treated) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer.

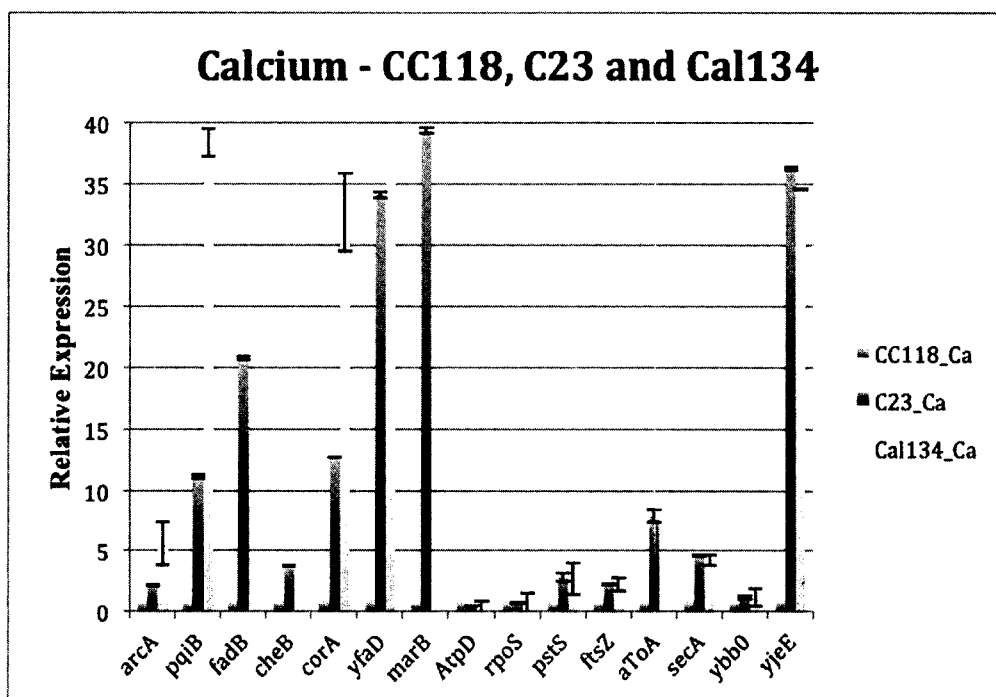


Figure 18. Relative expression of parental (CC118) versus mutant strains (PhoC23 and Cal134) under elevated calcium conditions (75 mM  $\text{Ca}^{2+}$ ). CC118 (calcium-treated) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer. The calcium-sensitive mutant Cal134 failed to yield any Ct values in qRT-PCR for *fadB*, *cheB*, *marB*, and *atoA* genes.

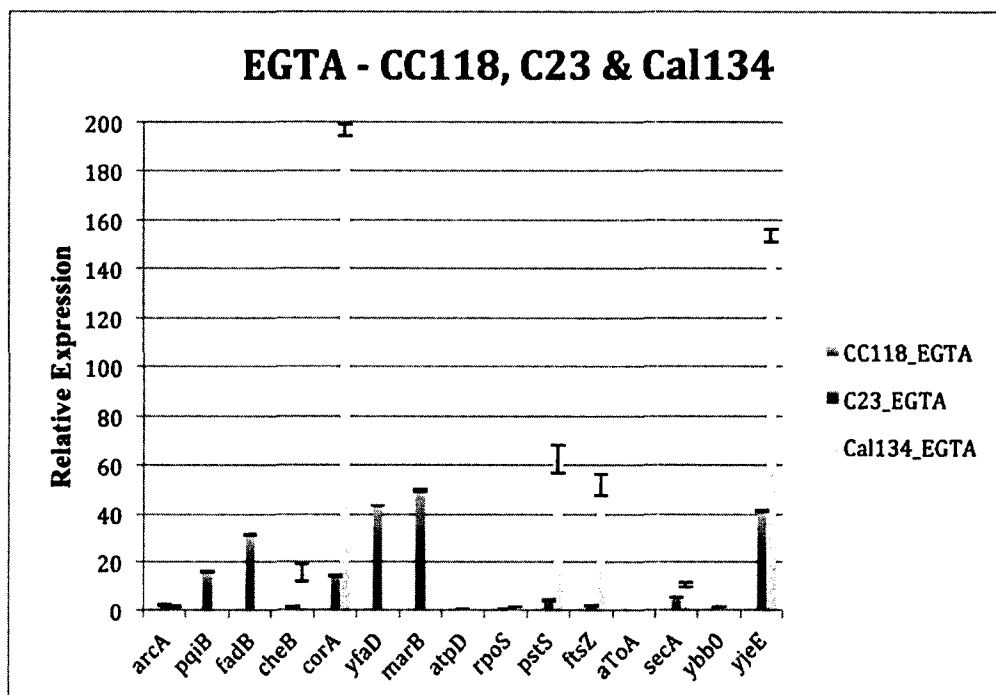


Figure 19. Relative expression of parental (CC118) versus mutant strains (PhoC23 and Cal134) under depleted calcium conditions (10 mM EGTA). CC118 (EGTA-treated) was used as the calibrator. The housekeeping gene *rpoB* was used as the normalizer. The calcium-sensitive mutant Cal134 failed to yield any Ct values in qRT-PCR for *pqiB*, *fadB*, *yfaD*, *marB*, *atoA*, and *ybbO* genes.

calcium conditions as gleaned from qRT-PCR (Figure, move to array section) and were close to the results obtained with Panorama *E. coli* macroarray experiments (Table 6).

### **secA and yjeE Expression**

Bioinformatics analysis of ATP-driven transporters or ATPases in the *E. coli* genome resulted in the identification of two essential genes, *secA* and *yjeE*, that likely code for a Ca<sup>2+</sup> efflux pathway (Naseem *et al*, 2009).

Both of these genes were upregulated (approx. 2- 3 fold) in CalC and CalD mutants in the presence of calcium and EGTA (Figures 16 and 17). The elevated calcium conditions resulted in 35-fold upregulation of *yjeE* in PhoC23 and Cal134 mutants (Figure 15). The *yjeE* gene was upregulated approx. 40-fold in PhoC23 and 150-fold in Cal134 under depleted calcium (EGTA) conditions (Figure 19). An overactive efflux system may conceivably be involved in these mutants in order to maintain calcium homeostasis.

### **fadB Expression**

*fadB* encodes the  $\alpha$  subunit of a multienzyme complex that is involved in the  $\beta$ -oxidation/degradation of fatty acids (Black and DiRusso, 1994). Mutants CalC and CalD showed 3-fold higher expression when in elevated calcium, Tnp*phoA* mutant PhoC23 exhibited 20-fold upregulation in calcium and 30-fold upregulation when grown in the presence of EGTA (Figures 18 and 19).

### **pstS, arcA, and rpoS Expression**

The periplasmic phosphate binding protein/phosphate transporter gene *pstS* expression was downregulated 2-fold in both elevated/depleted calcium conditions for both CalC and CalD mutants (Figure 16 and 17). However, with depleted calcium condition, *pstS* expression was upregulated 5-fold in PhoC23 and 60-fold in Cal134 (Figure 16). The wild-type MG1655 cells showed a downregulation of 5-fold in depleted calcium condition (Figure, move to array section).

The magnitude of upregulation (60-fold) in the case of Cal134 during depleted calcium condition is intriguing and begs the question whether phosphate starvation or stress response are somehow involved in calcium homeostasis processes in the cell. Phosphate limitation can subsequently induce *arcAB* (Marzan and Shimizu, 2011).

It is interesting to note that *arcA* is upregulated 5-fold in both CalC and CalD in elevated/depleted calcium conditions (Figures 16 and 17). In addition, *arcA* induction is evident in Tn*phoA* mutants PhoC23 (2-fold) and Cal134 (5-fold) in elevated calcium (Figure 18) but not in depleted calcium condition (Figure 19). Furthermore, increased expression of *rpoS* (8-fold in CalC and 10-fold in CalD) in elevated calcium condition (Figure 19) also may be indicative of a stress response. However, such a trend is not seen in the transposon mutants as *rpoS* level virtually remains unchanged when compared to the parental CC118 (Figures 18 and 19). There is a marginal but distinct increase in *rpoS* expression level in wild-type MG1655 with elevated and depleted calcium conditions as shown in Figure (Figure 12).

### *ftsZ* Expression

Transposon mutant Cal134 demonstrated 50-fold upregulation of *ftsZ* (essential cell division protein FtsZ) during depleted calcium condition (Figure 19). The cell division mutant was 10 to 20 times larger than the parent CC118 and DAPI staining indicated exhibited condensed chromosomes within the cell (Figure 4).

CalC and CalD mutants had 2-fold higher expression of *ftsZ* when grown in the presence of calcium (Figure 16), but addition of EGTA did not result in any changes between the parental KBT001 and the mutants (Figure 17). Expression of this cell division gene in MG1655 was downregulated 2-fold in both elevated and depleted calcium conditions (Figure, move to array section).

Calcium's role in cell division has been studied to a brief extent. Cytoplasmic calcium levels increase during cell division as observed with electron probe microanalysis and x-ray mapping of *E. coli* (Chang and Somlyo, 1986). Prokaryotic cell cycle events are thought to be regulated through a single  $\text{Ca}^{2+}$  flux (Norris and Holland, 1988).

FtsZ assembly from a monomer to oligomer is a magnesium-dependent process, requires guanine nucleotides and involves GTP hydrolysis (Mukherjee and Lutkenhaus, 1992). FtsZ has also been shown to have in vitro calcium-stimulated polymerization and GTPase activity (Yu and Margolin, 1997). In my cell division mutant Cal134, overexpression of *ftsZ* can explain the cell division defect as evidenced under the microscope (Figure 2).



### **pqiB Expression**

Paraquat, a superoxide radical-generating agent, induces *pqiB* gene and it is regulated by the *soxRS* locus (Koh and Roe, 1995). Elevated (Figure 18) and depleted (Figure 19) calcium conditions upregulated *pqiB* in PhoC23 (10-fold) while Cal134 demonstrated approx. 40-fold increase in *ftsZ* expression in the presence of elevated calcium (Figure 18). The possible role of calcium homeostasis in oxidative stress during growth in elevated extracellular calcium condition is interesting to note. In contrast to the mutants, the wild type MG1655 showed downregulation (3 – 5-fold) of *pqiB* gene in both elevated and depleted calcium conditions (Figure 12).

### **corA Expression**

The magnesium/nickel/cobalt transporter gene *corA* was upregulated 180-fold in Cal134 and 15-fold in PhoC23 during depleted calcium condition (Figure 19). These mutants also exhibited overexpression (approx. 12-fold for PhoC23 and 32-fold for Cal134) when exposed to high calcium (Figure 18). As mentioned in Figure 5, calcium-sensitive *TnphoA* mutants were also sensitive to other divalent cations.

The *calA* mutation that confers sensitivity to calcium as described by Brey and Rosen (1979) was in a strain that also harbored a mutation in the *corA* locus. The *corA* mutants are sensitive to calcium suggesting that *corA* may have a role in calcium access to the interior of the cell (Park and Lusk, 1976). I would speculate the cytosolic free calcium level in the transposon mutant Cal134 to be significantly different from wild type

parent or PhoC23. Depletion of calcium seems to have a drastic effect on Cal134 as manifested by the dramatic upregulation of *corA*.

### **atoA Expression**

The qRT-PCR results exhibited a 10-fold and 4-fold upregulation of *atoA* under elevated calcium conditions in the mutants CalC and CalD, respectively (Figure 16).

Theodorou and Kyriakidis (2009) investigated the involvement of external  $\text{Ca}^{2+}$  on cPHB [complexed poly-(R)-3-hydroxybutyrate] biosynthesis in *E. coli*. The synthesis of cPHB is regulated by the AtoS-AtoC two-component system that in turn regulates the expression of the *atoDAEB* operon. In their study, growth of *E. coli* in the presence of increased calcium (0mM, 0.25mM, 0.50mM, 1.0mM, 5.0mM) resulted in concentration-dependent induction of cPHB biosynthesis. Maximal cPHB levels accumulated at higher calcium concentrations (2.5mM). Addition of EGTA downregulated cPHB biosynthesis but EGTA-mediated down-regulation of cPHB biosynthesis was circumvented by the addition of calcium and magnesium (Theodorou and Kyriakidis, 2009).

The mutants employed in my study, namely, CalC and CalD have cytoplasmic free calcium concentration of 1130 nM and 410 nM, respectively (Tisa and Adler, 1995a). During my qRT-PCR experiments, CalC in elevated extracellular calcium condition exhibited 10-fold induction of *atoA* (Figure 16). Similarly, CalD with intracellular free calcium of 410 nM exhibited 4-fold upregulation of *atoA* (Figure 16). Treatment with EGTA resulted in roughly 2-fold upregulation in these mutants (Figure 17).

Cytoplasmic Ca<sup>2+</sup> levels are not regulated in the calC and calD mutants (Tisa and Adler, 1995a). Growth of these mutants in the presence of EGTA had lower cytoplasmic Ca<sup>2+</sup> levels than those cells grown in the absence of EGTA. The addition of calcium to the growth medium resulted in elevated levels of cytoplasmic Ca<sup>2+</sup> levels for these two cal mutants. It will be very interesting to employ calC mutant (with cytoplasmic free calcium concentration of 1130 nM) to further characterize the involvement of calcium homeostasis during cPHB production. As PHB is a viable polymer of tremendous importance in industry, elucidating the correlation between calcium homeostasis and PHB synthesis may lead to construction of better PHB-producing strains.

### **yfaD and ybbO Expression**

The expression of *yfaD* that codes for a conserved protein (unknown function) was upregulated 35–40 fold in both elevated and depleted calcium conditions in transposon mutant PhoC23 (Figures 18 and 19). Cal134 exhibited approx. 38-fold upregulation under elevated calcium condition. However, *yfaD* was repressed between 5 – 10-fold in MG1655 under elevated and depleted calcium conditions (Figure, move to array section).

Another gene *ybbO* (unknown function) was upregulated approx. 9-fold in CalC mutant and 4-fold in CalD mutant under elevated calcium conditions (Figure 16). These mutants exhibited almost 2-fold induction under depleted calcium conditions (Figure 17). While it is very likely that these genes are under calcium regulation as evidenced by

changes in gene expression in elevated or depleted calcium, we have very little information available to assign any biological function quite yet.

### ***cheB* Expression**

As mentioned before, *calC* and *calD* mutants have high levels of cytoplasmic calcium. Both of the *cal* mutants are also defective in chemotaxis (Tisa and Adler, 1995a). During chemotaxis, chemoreceptors are controlled by methylation and demethylation. A specific protein methylesterase (methyl-accepting chemotaxis protein-glutamate methylesterase), the product of the *cheB* gene, catalyzes the demethylation reaction.

My qRT-PCR results showed the gene *cheB* slightly upregulated in elevated calcium condition in both *calC* and *calD* mutants (Figure 16). However, in depleted calcium condition the gene was downregulated in both mutants (Figure 17).

### **Future Directions**

It seems likely that calcium homeostasis represents a dynamic state with hitherto unknown regulatory activity throughout *E. coli* genome. While it is relatively non-remarkable to witness the cell division defect in calcium-sensitive mutants because of a priori knowledge of calcium involvement in FtsZ polymerization, it is quite intriguing to see calcium's involvement in stress response. Whether it is a direct or indirect regulatory process that culminates in the upregulation of *marB* in all calcium-sensitive mutants, or approx.10-fold induction of *rpoS* in elevated extracellular calcium condition in *calC* or

calD mutants – many questions are raised regarding calcium's role in stress response when looking at the quantitative qRT-PCR data. A systematic approach that utilizes the mutants, incorporates accurate measurement of cytoplasmic free calcium, encompasses microarray studies with subsequent qRT-PCR validation of a more robust subset of genes is warranted. Also interesting is the fact that there is no calcium influx gene that is identified yet. A greater understanding of the role of calcium in PHB synthesis and perhaps calcium sequestration within the cell may shed light on more interesting details of calcium homeostasis in bacterial cells.

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